Characterization of *Bacillus* and its lipopeptides for controlling fungal phytopathogens and enhancing agriculture output

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بسم الله الرحمن الرحيم

DECLERATION

I, Munira Quddus certify that research work titled "Characterization of *Bacillus* and its lipopeptides for controlling fungal phytopathogens and enhancing agriculture output" is my own work. The work has not been presented elsewhere for assessment. Where material has been used from other sources it has been properly acknowledge/referred.

Munira Quddus

CERTIFICATE

This thesis submitted by Ms. Munira Quddus to the Department of Microbiology, Faculty of Biological Sciences, Quaid-i-Azam University, Islamabad, Pakistan, is accepted in its present form as satisfying the requirement for the degree of Masters of Philosophy in Microbiology.

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List of Acronyms /Abbreviations

PGPR	Plant Growth Promoting Rhizobacteria
PSB	Phosphate Solubilizing Bacteria
BCA	Biological Control Agent
BHI	Brain heart infusion
DNA	Deoxyribonucleic Acid
EU	European Union
Fig	Figure
FAO	Food and Agriculture Organization
GDP	Gross Domestic Product
GRAS	Generally Recognized as Safe
g	Gram
IRRI	International Rice Research Institute
L	Liter
µg	Microgram
µl	Microliter
mg	Milligram
ml	Milliliter
PACRA	Pakistan Credit Rating Agency Limited
PDA	Potato Dextrose Agar
QAU	Quaid-i-Azam University
SDA	Sabouraud Dextrose Agar
WHO	World Health Organization

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Abstract

The agricultural productivity loss due to microbial phytopathogens has been a serious global issue. The present study was designed to evaluate soil-borne *Bacillus* strains for their biocontrol potential against various important fungal pathogens and for promoting plant growth. The bioactivity and PGPR profile of isolated *Bacillus* strains (MQ7 and MQ8) was determined against *Aspergillus fumigatus, Aspergillus flavus, Aspergillus niger and Fusarium oxysporum* using different in vitro techniques. Crude antifungal metabolites were extracted from the *Bacillus* MQ7 and MQ8 were characterized using FT-IR. Finally, green greenhouse experiments were conducted on the *Zea maize* plant to assess the antifungal and PGPR activity of *Bacillus* strains and their comparison with commercially available fungicides (Pyranazole and Fosetyl-Aluminium). The results indicated that the strain MQ7 exhibited excellent anti-fungal activity against *Aspergillus flavus*, *Aspergillus niger and Fusarium oxysporum* with inhibitory zones of 55mm, 56mm and 23mm respectively. In the case of strain MQ8, 35mm, 48mm, and 35mm of the zones were recorded for the aforementioned fungal isolates. The antifungal activity of the *Bacillus* strain MQ7 was comparatively higher than the strain MQ8 i.e. 50%±0.5 for *F. oxysporum*, and for *A. flavus* 28.71%±0.25. In-vivo experiments on *Zea maize* showed increase in plant growth up to 10.13% and 5.8% in the presence of strain MQ7 and MQ8 respectively, in comparison to the positive control. Based on the results, it can be concluded that *Bacillus* strains MQ7 and MQ8 can produce secondary metabolites with excellent antifungal activity and suppress the incidence of fungal infections under greenhouse conditions. In addition, the aforementioned strains effectively enhanced the growth of the *Zea maize* plants. Therefore, the *Bacillus* MQ7 and MQ8 could be effective biological resources as alternative biopesticides and biofertilizers and an important tool for sustainable agriculture.

1. Introduction

The continuous increase in global population will unavoidably result in depletion of resources, particularly food, water and energy. Gap in the demand and supply have a detrimental effect on the food supply chain. Providing food for the 9 billion people by 2050 will be the key challenge (Neupane et al., 2022). Scientists around the globe are looking for innovative methods to increase food production and avoid food losses especially in the agriculture sector owing to changing climate and invading phytopathogens. It has been witnessed that perturbations of biotic and abiotic factors have been leading to a reduction in current agriculture production. Almost 20-40% of agricultural productivity loss is due to the attack of invading pathogens and pests including bacteria, fungi, viruses etc (Shahzad et al., 2021). Contemporary methods to manage agricultural productivity losses include; the use of agro-chemicals and synthetic formulations however, these methods are associated with various health risks in the all-life forms (Bhanse et al., 2022). Considering the ongoing quest for food security and safety, it has become inevitable to design new strategies for coping with these issues

Plant diseases, which are caused by different microorganisms such as bacteria, fungi, viruses, protozoa, and nematodes, have a negative impact on agricultural production and yield losses. Pathogenic fungi cause 10–20% losses in agricultural crop productivity (Gurr, 2023). Some types of fungi affect all plants, and each of the pathogenic fungus can attack a single or various types of plant species. About 13,000 species of fungi are responsible for plant diseases. *Fusarium* species are important plant pathogens that cause a variety of plant diseases, including; wilts, head blights, fruit and root rots. *F. graminearum* stands out as a significant fungal phytopathogen, producing mycotoxins such as deoxynivalenol (DON) and trichothecenes like nivalenol (NIV), as well as its derivatives, 3- and 15-acetyldeoxynivalenol (3-ADON, 15-ADON). These mycotoxins cause infections in cereal crops and have negative effects like immunosuppression and neurological problems in animals. (Asad, 2022). A fungus called *Rhizoctonia solani* causes root rot and damping off in over 2000 plant species (Ezrari et al., 2021). These ailments are not only difficult to treat but also cause significant crop losses. To lessen these incidences of plant diseases several control measures have been employed including; the use of chemical pesticides,

crop rotation, and cultivars that are less susceptible however, their effectiveness is typically insufficient because soil-borne pathogens often survive and develop resistance.

The use of chemical pesticides to deal with infectious diseases has resulted in a slew of environmental and health problems. Pesticides and other agrochemical elements are thought to be responsible for up to 14% of all occupational injuries, according to the reports of the International Labor Organization (Khatri et al., 2023). The growing demand for food has directly resulted in the additional usage of 2 million tons of pesticides each year in Europe, the United States and the rest of the world. Only an estimated 0.1% of the three million tons of the active chemicals in these pesticides are helpful in the protection of crops while the remaining (99.9%) is wasted and becomes part of the environment (i.e., soil, air and water), due to ineffective application methods, photodegradation and deposition (Medina et al., 2019). Traditional pesticides are no longer effective against plant pathogenic bacteria and fungi owing to the development of resistance. Considering the adverse effects of chemicals, the hunt for more safe and effective compounds, particularly for pathogenic fungus of the plants has been increased (Gill and Garg, 2014).

Aiming to improve crop yield and control plant diseases in an integrated crop management system (ICMS), research is now focused on environmentally acceptable alternatives. The employment of beneficial organisms to lessen the negative impacts of plant diseases and encourage good responses from the plant is known as biological control, and it is a crucial part of an ICMS. The most typical method of biological control involves choosing antagonistic microorganisms, deciphering their mechanisms of action, and developing biocontrol preparations (El-zik and Frisbie, 2018; Lahlali et al., 2022). Among the most researched biocontrol agents, or biopesticides that help to inhibit plant diseases through competition and/or antagonism, are *Bacillus* species. Antifungal *Bacillus* species have various benefits over other organisms, including the capability to produce endospores and the ability to survive severe temperature, pH and osmotic conditions. *Bacillus* species have been reported to effectively colonize the surface of roots, enhance the growth of plants and also cause lysis of the fungal cell wall (El-bendary et al., 2016). A diverse variety of strategies are employed to inhibit the growth of pathogens by *Bacillus*, including competing for space and nutrients, synthesizing hydrolytic enzymes, antibiotics, and inducing systemic resistance. It is noteworthy that some *Bacillus* species, like *B.*

subtilis and *B. amyloliquefaciens*, devote up to 8% of their genetic makeup to the synthesis of a wide range of antimicrobial compounds. These include non-ribosomally synthesized polyketides and lipopeptides. *Bacillus* spp. can also function as biostimulators or biofertilizers either by promoting the uptake of specific nutrients from the surroundings (siderophore production, phosphate solubilization and nitrogen fixation) or by supplying the plant with certain compounds (Synthesis of plant hormones) (Bolivar-Anillo et al., 2021).

Globally, the emergence of phytopathogens resistant to traditional treatments as well as the widespread use of chemical fertilizers, fungicides and pesticides for crop protection has led to serious environmental and health complications (Ali et al., 2021). The necessity of looking into alternate management techniques that are successful while minimizing ecological damage cannot be stressed in light of these difficulties. Thus, *Bacillus* spp. serves as an alternative to agrochemicals or synthetic pesticides and fertilizers that are used to stimulate plant development. The advantages of *Bacillus* spp. on plant growth and overall production have been proven in a variety of crops, including maize, wheat, pepper, cucumber, soybean, potato, common bean, tomato, sunflower, and many others. Variability between results obtained in the laboratory, greenhouse, and field limits the application of *Bacillus* spp. to boost the productivity of field and vegetable crops (Chausali and Saxena, 2022). Therefore, current research is an attempt to isolate beneficial soil microorganisms to increase plant growth and metabolic potential to reduce productivity losses caused by plant pathogens.

Aim and objectives

Aim

The aim of the current study is "Characterization of *Bacillus* and its lipopeptides for controlling fungal phytopathogens and enhancing agriculture output". The following are the objectives;

Objectives

1. Sampling, isolation and screening of *Bacillus* strains capable of producing cyclic lipopeptides.
2. Anti-fungal activity of *Bacillus* strains against four economically important fungal phytopathogens.
3. Characterization of lipopeptides and detection of biosynthetic genes (surfactin, fengycin & iturin) in isolated *Bacillus* strain
4. Green house experiments of anti-fungal and PGPR activity of selected *Bacillus* strains

2. Literature review

Plant diseases cause massive losses in agricultural output by reducing crop yields and quality, resulting in significant economic loss (Rashad and Moussa, 2020). Plant disease losses are expected to be more than 15% for crops that are unprotected globally (Chatterjee et al., 2016; Asad, 2022). More importantly, fungal phytopathogens cause more than 70% of these diseases (Liu et al., 2017). *Aspergillus, Alternaria, Fusarium, Cladosporium, Botrytis, Verticillium, Rhizoctonia* and *Pythium* are the most commonly found fungal phytopathogens (Li and Chen, 2019; Krylov et al., 2018; Doehlemann et al., 2017; Djonovic et al., 2007; Zhang et al., 2016). Chemical fungicides are the most common option for managing phytopathogens in recent decades, with yearly consumption topping one million tonnes. However, the ongoing climate change situation and agricultural pollution are spurring the development of environmentally friendly agricultural products, including safe plant protection products (Lahlali et al., 2022; Mhatre et al., 2018; Khan et al., 2022; Köhl et al., 2019)

Biological management with beneficial bacterial strains is an environmentally acceptable approach to mitigating plant phytopathogens (Pascale et al., 2020). Commonly known organisms in the environment are bacteria, which are not uniformly dispersed. For instance, root exudates, which contain substances like amino acids, organic acids or sugars that serve as an energy source for bacteria, can cause the density of bacteria around plant roots to be significantly higher than the density found in the soil overall (Basu et al., 2021; Khan et al., 2021; Bhat et al., 2023). Importantly, a variety of bacterial strains that are present in or near the rhizosphere soil and can promote plant growth are known as plant growth-promoting bacteria (PGPB). Additionally, according to Morales-Cedeo et al. (2002), endophytic bacteria are also present in PGPB. Directly or indirectly, PGPB can stimulate plant growth. Direct plant growth promotion traits include, e.g., atmospheric nitrogen fixation, gibberellins production, indolyl-3-acetic acid (IAA) production, cytokinins production, 1-aminocyclopropane-1-carboxylic acid deaminase (ACC), and phosphorus solubilization (Kaur et al., 2022; Sarmiento-López et al., 2022). In the meantime, biocontrol traits are characterized as an indirect mechanism of plant growth enhancement, for example, the production of antibiotics such as cyclic lipopeptides, glucanase, chitinase, and the induction of induced systemic resistance (ISR) (Shahid et al., 2021; Mirskaya et al., 2022). The aforementioned solutions are in line with the modern trends set forth by the

EU's strategic programmes and the tenets of the EU Biodiversity Strategy for 2030 and the European Green Deal (EGD), which emphasise the importance of agroecology development, agricultural biologicalization and an expansion of the area used for ecological crops (Montanarella and Panagos, 2021).

The bacteria in the genus *Bacillus* are among the most intriguing in terms of agricultural applications. According to Pudova et al. (2022), Nicholson (2002), Yakovleva et al. (2022), it primarily consists of Gram-positive, spore-forming bacteria that are widely distributed in a variety of habitats, including soil. Importantly, *Bacillus* spp. strains have notable resilience to environmental challenges such as drought, UV light, irradiation, and poor nutrient availability, which also boosts their capacity to promote plant development (Nicholson et al., 2000). Numerous species of the genus *Bacillus* have been recognized as PGPB thus far, including *B. subtilis* (Siahmoshteh et al., 2018), *B. licheniformis* (Gomaa, 2012), *B. cereus* (Chauhan et., 2016), *B. megaterium* (Mannaa and Kim, 2018), *B. amyloliquefaciens* (Siahmoshteh et al., 2018), etc. *B. subtilis* is the *Bacillus* spp. species that have been most thoroughly examined in terms of biocontrol agents. Numerous preparations in the market contain it in the formulation due to its properties, which include ISR induction and the production of hydrolytic enzymes or lipopeptides (Samaras et al., 2021). It has already been commercialised as a biocontrol agent for fungal phytopathogen.

2.1. Crop loss analysis

According to estimates, total productivity loss owing to phytopathogens is around 15-20%. Pests cause around 50% of crop losses in wheat, 80% in cotton, 31% in maize, 30% in soybeans, 37% in rice, and 40% in potatoes. It is believed that roughly 1,300 species of fungi are also responsible for various crop diseases. The annual loss of several important crops, such as potatoes, wheat, maize, various vegetables and fruits, ranges from 11 to 14%. This loss equates to an annual loss of $80 billion. Viruses are also well recognized to harm plants and cause about 41% loss (International Committee on Taxonomy of Viruses). Furthermore, approximately 100 various types of bacteria have also been reported to cause bacterial infections in plants, accounting for 16% of crop loss (Oerke, 2006).

Pakistan is an agricultural country and reportedly one of the world's top producers and suppliers of food and crops. Pakistan is ranked 8th globally in terms of agricultural output on the list of countries by GDP sector composition. According to the Pakistan Bureau of Statistics, the agriculture sector contributes up to 24% of Pakistan's GDP. Although many factors contribute to the loss in overall productivity, however, soil-born fungal phytopathogens are one of the major issues to deal with in modern agriculture. Recently in 2023, a catastrophic situation was observed in Sawabi district, Pakistan where the wheat crop was attacked by fungal disease, leaf rust. The agriculture department declared an emergency state. Leaf rust was found to spread through crops like cancer, and if it was not controlled, the harm might be irreparable (DAWN, 2023). The current study seeks to solve productivity loss caused by pathogenic microorganisms in reducing agricultural output.

2.2. Development of Fungal infections in plants

A disease cycle is a series of sequential processes that occur when a pathogen interacts with a host and results in the development and continuation of disease. The phases of a general illness cycle are as follows (Zeilinger *et al*., 2016):

i. **Spread and contact:** Environmental methods e.g., water, wind, insects, and active development, as in the case of many root-infecting fungi, are used to disperse fungi and bring the fungus into active contact with the appropriate host.
ii. **Pre-penetration:** It includes the adhesion of the pathogen to the structures of host and the recognition processes prompted by both host and environmental signals.
iii. **Pathogenic entry:** Pathogens enter plants through the natural holes and wounds, or their direct penetration through specialized penetration structures like appressoria or insect-inflicted wounds.
iv. **Invasion and infection:** The step in which the pathogen establishes active contact with the cells of the host and spreads from one cell to the other, causing symptoms to appear**.**
v. **Reproduction:** An enormous quantity of spores is produced from the tissues of the infected host during reproduction.
vi. **Spore dissemination:** Spores are dispersed from the site of a reproduction of fungus to the other vulnerable plants.

vii. **Dormancy:** Dormancy aids in the survival of pathogens in harsh environments.

2.3. Mode of infection by pathogens

- **Cell wall-destroying enzymes:** These specific enzymes cause the breakdown of the cell wall of plant cells and the release of nutrients present in them.
- **Toxins:** Toxins may be host-specific which causes damage to their specific host or non-specific which can cause damage to all susceptible plants.
- **Effector proteins:** The effector proteins are the proteins which are directly released into the outside environment through the type-3 secretion pathway. Some effector proteins have been shown to cause the suppression of the host's defence mechanisms. This could entail lowering the plant's internal signalling processes or lowering the production of phytochemicals. This function is known to be performed by fungi, bacteria, and oomycetes.
- **Spores:** Phytopathogenic fungi can infect host plants by their spores. Spores stick to the cuticular layer on the host plant's leaves and stems initially. The highly infectious spores are required to be carried from the source pathogen and it happens through vectors e.g., wind, water, insects and humans. When suitable conditions exist, the spore will develop a germ tube, which is a modified hypha. This germ tube develops into the appressorium, which produces melanin-containing cell walls to increase turgor. The appressorium pressurizes the cuticular layer like a hardened penetrating peg once sufficient turgor pressure has been accumulated. The secretion of enzymes capable of destroying cell walls, from this appressorium also aids this process. When this peg penetrates the tissues of the host, it develops a haustorium, which is a specialized hypha. This haustorium could penetrate and feed on the cells or persist intercellularly within the host, depending on the pathogen's life cycle (Akram et al., 2023).

2.4. Strategy to mitigate crop productivity loss

Pathogen control is frequently dependent on the adoption of cultivars having resistance to fungal infections, rotation of crops to prevent the buildup of pathogen populations and proper management of soil e.g., ploughing, the addition of organic matter and liming. The build-up of microbial communities that are hostile to pathogens can lead to what is called a take-all decline,

in which the severity of a disease is reduced after various seasons due to persistent growth of the same wheat crop. The control by chemicals is scarcely possible due to the ability of soil to buffer bioactive substances and the microflora's quick breakdown of such compounds (Manik *et al*., 2019).

Pests and diseases can be controlled using a variety of methods. Biological and chemical approaches are the most common and widely used, yet there are significant distinctions between them.

2.4.1. Chemical control of Phytopathogens

To manage diseases, weeds and pests' various chemical pesticides are frequently used. The management of pests by chemicals uses chemical compounds that are poisonous to the pest species. Plant protection compounds are chemical insecticides, employed to protect plants from weeds and pests. It's of course critical that the plants to be protected do not become toxic as a result of the protective products (Oerke. 2006).

Pesticides can be divided into 5 groups based on their reasons for use. Fungicides are included in the first of these groups and they have strong efficacy against fungi. Herbicides, on the other hand, are used to kill weeds. The herbicides are absorbed by weed's roots or leaves, leading it its death. There are various insecticides, which, as their name implies, kill hazardous pests, and acaricides, which protect plants against mites. Finally, nematodes that harm plants can be controlled with nematicides.

2.4.1.1 Advantages and drawbacks of the chemical control method

Chemical pesticides are widely used due to their low cost, easy availability, simplicity of application, and effectiveness and chemical stability. The chemical insecticides are often rapid-acting, which reduces crop harm.

Chemical pesticides have many significant disadvantages, although they continue to be widely sold and utilized. To begin with, chemical pesticides are frequently hazardous to organisms other than those for which they were designed. Chemical pesticides can be categorized into selective and non-selective types. Non-selective compounds are dangerous since they destroy all types of

organisms, even those that are beneficial and harmless. Herbicides, for instance, can obliterate both the broad leafy weeds as well as grasses. Because these compounds kill practically all of the vegetation, they are non-selective (Arjjumend et al., 2021).

Pesticides with a narrower spectrum of action are known as selective pesticides. They only kill the pest, illness, or weed in question; other organisms are unaffected. A weed-killer compound that can only kill broadleaf weedy plants is an example. Because it cannot finish off the grass, it could be utilized in the lawns. Because all of these chemical products have selective activity and can control a defined range of pests.

Chemical insecticides also have the problem of resistance. Pesticides are effective only on a definite organism and also for a brief time duration. The organisms can develop immunity to the material, meaning it is ineffective on them. These creatures evolve and become resistant as a result of their mutations. This means that more insecticides will have to be employed to keep them under control (Arjjumendet al., 2021).

The growth of debt is a third disadvantage. Chemicals are transferred up in a food chain when a plant sprayed with pesticides is consumed by an animal which is then eaten up by its predator and so on. This leads to the accumulation of pesticides among the animals that are towards the top of a food chain e.g., humans and predators. Thus, these animals are at the risk of poisoning. The last and most serious concern is the pesticide residues that are retained on crops. Because residue can be eaten up on vegetables and fruits, crops should not be sprayed too close to harvesting. Instead, pesticide residues could seep into soil or go into groundwater, contaminating the water which is then used up by animals.

In summary, various strategies are used to reduce pesticide's negative environmental effects: use only selective pesticides which kill only harmful organisms and use a pesticide which can break down easily and care should be exercised while spraying the crops (Arjjumend et al., 2021).

2.4.2. Biological control

Biological control can be categorized into;

- **Macrobials**
- **Microbials**
- **Biochemicals**

2.4.2.1. Biological control by parasites or other natural predators

Biological control is based on the assumption that a pest can be controlled by other parasites or its natural predators. As a result, the natural predators and enemies were initially introduced to combat the given pests. These natural parasites and predators were introduced in extremely limited numbers at first, but once they were established, they proved to be effective over time. Inoculation is another name for this practice. Inundation occurs when a natural predator is introduced regularly. Predators and parasites can also be beneficial microbial creatures (Hajek and Eilenberg, 2018). The term parasite is used to refer to an organism which exists at the cost of its host e.g., as parasitic wasp larvae that reside within the larvae of whitefly and consume whitefly larvae from inside. Predators, e.g., ladybirds consume the aphids, are the species simply feeding on the other organisms for their nourishment.

2.4.2.2. Biological control by micro-organisms (the microbial)

A variety of useful microbes could be utilized to boost the health of plants and help combat diseases and pests. Bacteria, fungi, and other microorganisms can lead to these consequences by competing for limited space and nutrients, producing antibiotic compounds or simply by eating other hazardous microbes. Microorganisms could even be employed to protect plants by making them healthier and tougher. Pests and illnesses have little chance to affect the plants when this happens. Such control of pests is usually unseen. *Bacillus subtilis* and *Trichoderma* are the two examples of most commonly utilized microbials (Puopolo et al., 2018).

2.4.2.3. Biological control by the pheromones and chemicals of natural origin (biochemicals)

There are extensive natural resources and different pheromones that may be utilized to manage various plant diseases as well aspects. Plant extracts, plant hormones and vitamins belong to this category. These also work as preventative measures to make sure that the plants are strong and

healthy. Pheromones are the chemicals which are used to attract the insect pests into a trap. The most common forms are aggregating pheromones and the sex pheromones (Sarwar, 2015).

2.4.3. Advantages and disadvantages of biocontrol methods

Biological control, like chemical control, offers both benefits and drawbacks. The first benefit is that the natural foe can establish itself, resulting in long-term consequences. Pests cannot develop resistance to being eaten, therefore the chance of resistance is significantly reduced.

Natural pest control is highly targeted, making it an excellent method of controlling specific pests. The downsides of biological control include the possibility that natural adversaries will flee. This issue could be handled in the greenhouses, but difficult to handle in the fields. It takes time to spread out over a broader plot area. Second, pests are never totally eradicated since the natural opponent must survive, and as a result, they will never eradicate the entire population. Finally, they cannot be used before the pest has appeared, which implies that crops will suffer some damage (Arjjumend et al, 2021).

Some biological uses aren't entirely risk-free. Even though those are natural products, they may affect creatures beyond those targeted. A crop can be destroyed by its natural enemy, especially when very large numbers are needed for the suppression of a given pest. Natural predators and enemies have a minor dramatic impact than chemical control. Because insects have already spread to far-flung areas, if this biological control technique fails, an increased dose of chemical pesticides may be required. Finally, besides removing the diseased plants, there are almost no natural techniques for the control of viruses. Biological control just like chemical control, is always evolving just as new species of pests (fungi, insects and bacteria) emerge and these organisms rapidly mutate. Plant protection compounds, like pesticides, are categorised as biological control agents that use compounds of natural origin. They must meet the same stringent requirements as pesticides. As a result, this class of "plant protection products" might be quite costly (Arjjumend et al., 2021).

2.5. Mechanisms of Biological Control by *Bacillus*

2.5.1. Antibiotic production by *Bacillus*

Bacillus spp.'s antagonistic behaviours are frequently linked to producing secondary metabolites with antibiotic capabilities. Cyclic lipopeptides, peptides, and polyketides are examples of low molecular weight peptides that are produced either ribosomally (as in bacteriocins) or non-ribosomally (as in polyketides). Numerous bacteria create ribosomally synthesised peptides called bacteriocins, that may be efficient against pathogenic and resistant bacteria (Zou et al., 2018). Bacteriocins affect their target cells by causing pores in the cell membrane or interfering with the formation of the cell wall (Juturu and Wu, 2018). Bacteriocins have a narrow spectrum of action and are typically focused against species that are identical to or nearly related to the producers. *Bacillus* species nevertheless display a wide range of antibacterial activity because they produce bacteriocins (Salazar et al., 2017). According to some reports, many *Bacillus* spp., such *as B. amyloliquefaciens, B. subtilis, B. cereus, B. thuringiensis,* and *B. coagulans*, produce bacteriocins and bacteriocin-like substances (BLSs), such as amylolysin, subtilin, amysin, subtilosin A, and subtilosin B (Abriouel et al., 2011). Bacteriocins and BLSs that have been isolated and characterised may be used in significant ways to control harmful bacteria biologically. But *Bacillus* species, which generate peptides and lipopeptides not made by ribosomes, have considerably more potent antibacterial properties (Fira et al., 2018).

The antibiotic compounds in *Bacillus* spp. that have been the subject of the most research are cyclic lipopeptides (LPs), which are well known for their antagonistic effect against a variety of plant pathogens (Stein, 2005). Large non-ribosomal peptide synthetases (NRPSs) are responsible for the synthesis of these peptides. The fundamental mechanisms of LPs' effects typically entail interaction with the target pathogens' cell membrane, altering its composition and permeability by rupture, solubilization, or the development of ion-conducting holes (Fira et al., 2018). Additionally proven is the interaction of LPs with intracellular components including DNA. According to recent studies, LPs influence *Bacillus* colonization and persistence in the rhizosphere and activate plant defence mechanisms (Han et al., 2015). Families of the surfactin, iturin, and fengycin cyclic LPs from *Bacillus* spp. are the most significant (Stein, 2005). Some other non-ribosomally produced LPs include bacitracins, polymyxins, tyrocidines, kurstakins and

gramicidins. Numerous investigations showed that *Bacillus spp*. produce a wide range of antibiotics, which have an antibacterial effect (Table 2.1).

Table 2.1: Biocontrol mechanisms used *Bacillus* against fungal phytopathogens to treat plant diseases

***Bacillus* species**	**Target pathogens**	**Mechanisms**	**Plant diseases**	**Reference**
Bacillus subtilis	*Bipolaris sorokiniana*	Lytic enzymes, siderophores production	Spot-blotch disease of wheat	Villa et al., 2019
Bacillus subtilis	*Fusarium oxysporum*	Siderophores production	Pepper Wilt	Yu et al., 2011
Bacillus velezensis	*Botrytis cinerea*	Cellulase, Glucanase Chitinase, Protease,	Gray mold in pepper	Jiang et al., 2018
Bacillus sp	*Fusarium verticillioides*	siderophores glucanase, Protease, chitinase,	Ear rot and Stalk of maize	Douriet et al., 2018
Bacillus amyloliquefaciens	*Clavibacter michiganensis*	chitinase cellulase, protease, lipase, Siderophores	Bacterial canker disease of tomato	Gautam et al., 2019
Bacillus amyloliquefaciens	*Fusarium oxysporum* f. sp. *Lycopersici*	Protease production	Wilt disease in tomato	Guleria et al., 2016
Bacillus subtilis	*Rhizoctonia solani*	Chitinase production	black scurf and Stem canker disease of potato	Saber et al., 2015
Bacillus licheniformis	*Magnaporthe grisea*	Surfactin production	Blast disease in rice	Tendulkar et al., 2007
Bacillus subtilis	*Gaeumannomyces graminis* var. *tritici*	bacillomycin, surfactin, Iturin, difficidin, plipastatin	Take-all disease of wheat	

Bacillus amyloliquefaciens, Bacillus pumilus	*Pseudomonas syringae* pv. *aptata*	Lipopeptides production	Leaf spot in sugar beet	Nikolic et al., 2019
Bacillus subtilis	*Pythium ultimum*	Fengycin, Iturin	Damping-off disease in bean	Ongena et al., 2005
Bacillus subtilis	*Fusarium* spp.	Surfactin, Lytic enzymes	Clove rot disease in garlic	Bjelic et al., 2018
Bacillus amyloliquefaciens	*Verticillium dahliae*	Iturin production	Cotton Wilt	Han et al., 2015
Bacillus amyloliquefaciens	*Fusarium oxysporum*, *Mucor* sp., *Fusarium avenaceum*, several pathogenic bacteria,	Surfactin, bacteriocins, fengycin	Various diseases of vegetable crops and field	Salazar et al., 2017

2.5.2. Lytic Enzymes of *Bacillus*

The hydrolytic enzymes chitinases, cellulases, glucanases, chitosanases, proteases and lipases which effectively hydrolyze the different key components of the bacterial and fungal cell wall structure, may also be responsible for *Bacillus* spp.'s antimicrobial action. Chitin, which is the second most prevalent naturally occurring polysaccharide after cellulose and the primary constituent of fungal cell wall, is broken down by chitinases, which are glycoside hydrolases (GHs) (Rathore and Gupta, 2015). Chitinases are generally made by bacteria to break down chitin for use as an energy source, while some chitinases have potential as biological control agents for a range of plant diseases brought on by phytopathogenic fungi. The hydrolytic breakdown of the β-1,4-glycosidic linkages in the chitin derivative chitosan is facilitated by GHs called chitosanases (Weikert et al., 2017). For the considerable carbon and nitrogen recycling, chitosanases are crucial. *Bacillus* spp. that produce chitosanase can be utilised as biocontrol agents to stop pathogen-caused plant infection because chitosan is also present in the cell walls of fungi (Seo et al., 2014). GHs called glucanases hydrolyze the glycosidic bonds found in α- and β-glucans. Although α-1,3-glucan is not a necessary component of the cell wall, it does play a significant role in some fungi's vegetative development and cell division, while, β-1,3-glucans is

the second most important component of the fungal cell wall after chitin. Cell wall glucans in fungi mostly serve a structural purpose, though they can also be broken down and used as nutrient. α-1,3-glucanase and β-1,3-glucanase can be found in abundance in *Bacillus* spp. *B. licheniformis, B. circulans, Bacillus brevis, Bacillus halodurans, and B. subtilis* have been reported to produce the enzymes (Planas, 2000). The skeleton of fungal cell walls is made up of cellulose, lipids, and proteins in addition to chitin and glucan. Thus, the cell wall lysis that takes place during pathogen-*Bacillus* interactions may be significantly influenced by bacterial cellulases, proteases and lipases (Guleria et al., 2016).

The activity of several enzymes is required for efficient cell wall breakdown. The hydrolytic activity of other enzymes, especially glucanases, occurs before or at the same time as chitinase activity. Maximum effectiveness may need combinations of hydrolytic enzymes with complementary mechanisms of action, and the right enzyme combinations may boost antifungal activity (Mardanova et al., 2016). Recent research has shown that *Bacillus* spp. can produce lytic enzymes to act as biocontrol agents (Table 2.1).

2.5.3. Siderophores by *Bacillus*

Some microbes and plants, particularly those that are iron-starved, create siderophores, which are non-ribosomal peptides that chelate metals and have low molecular weight (Khan et al., 2018). The biological activities of oxygen consumption, electron transfer, DNA, RNA synthesis, and enzymatic reactions all require the metal iron (Fe). Siderophores' main function is to chelate iron, which enables its extraction from minerals and organic compounds and solubilization in solution. The importance of siderophores in biological control is based on Fe competition, which lowers pathogen availability. Additionally, microbial siderophores can be reduced to contribute Fe to a plant's transport system or chelate iron from soils and engage in a ligand exchange with phytosiderophores, supplying plants with this crucial element to promote growth (Saha et al., 2016). Siderophores can bind several environmental metals in addition to Fe, serving as bioremediation agents in the process (Ahmed and Holmstrom, 2014).

2.5.4. Systematically induced disease resistance by *Bacillus*

Through defence mechanisms, plants adapt to ongoing pathogen exposure. After appropriate stimulation, a plant's ability to resist pathogens improves. Following preventive structural and histological alterations, preventive chemical compounds (phenols and other byproducts of secondary metabolism), and other preventive measures, infected plants increase their levels of signalling molecules that coordinate the activation of genes for appropriate synthesis (Pieterse et al., 2014).

Systemic acquired resistance (SAR), which develops as a result of a localised infection, is one of the plant defence mechanisms that can be started by external substances prior to infection (Chunyu et al., 2017). Inducing ISR in plants against several plant diseases has been done using both biotic and abiotic stimuli. ISR is induced by non-pathogenic rhizobacteria and primarily relies on the jasmonate (JA) and/or ethylene (ET) signalling pathways, whereas SAR is mediated by a salicylic acid (SA)-dependent mechanism. SAR also activates a collection of defence-related genes linked to the generation of pathogenesis-related proteins (PR), whereas ISR does not . ISR-mediated defences are much less effective than SAR-mediated ones. However, ISR and SAR work better when combined, showing that they can function additively to increase pathogen resistance (Niu et al., 2016).

Table 2.2: *Bacillus* as biocontrol agent against various fungal phytopathogens

***Bacillus* specie**	**Plant diseases**	**Target pathogen**	**Reference**
Bacillus sp	Downy mildew disese in sunflower	*Plasmopara halstedii*	Nandeeshkumar et al., 2008
Bacillus subtilis	Root rot disease in cucumber	*Fusarium oxysporum*	Chen et al., 2010
Bacillus sp	Root wilt and rot in soybean	*Fusarium oxysporum, Rhizoctonia solani*	Jain et al., 2017
Bacillus spp	Blast disease in rice	*Pyricularia oryzae*	Rais et al., 2017
Bacillus subtilis	Early and late blight disease in tomato	*Phytophthora infestans, Alternaria solani*	Chowdappa et al., 2013

Bacillus amyloliquefaciens, Bacillus subtilis	Root, ear and stalk rots in maize	*Fusarium moniliforme*	Gond et al., 2015
Bacillus amyloliquefaciens	Leaf spot disease in pepper	*Xanthomonas axonopodis* pv. *Vesicatoria*	Choi et al., 2014
Bacillus cereus	Gray mold disease of field and vegetable crops	*Botrytis cinerea*	Nie et al., 2017
Bacillus amyloliquifaciens	Wilt disease in tomato	Potato virus Y, Tomato spotted wilt virus	Beris et al., 2018

2.6. Mechanisms of plant growth promotion by *Bacillus*

2.6.1. Nutrient availability

Diversity of metabolites produced by *Bacillus* spp. can improve the availability of nutrients to plants, hence enhancing plant growth and output. The majority of the nutrients that plants need are provided by mineral fertilisation, a process that not only harms the environment severely but also results in enormous economic losses. A plausible strategy to lessen the detrimental effects of synthetic fertilisers without sacrificing food safety is to employ biofertilizers that contain P-solubilizing and/or N2-fixing *Bacillus* spp. (Bhattacharayya et al., 2016). Different plants' ability to absorb nutrients and then promote their own growth is closely correlated with P-solubilizing and N2-fixing *Bacillus* spp.

Phosphorus (P) plays a direct role in plant growth. But a significant amount of P (more than 80%) is fixed in the soil and unable to be absorbed by plants because of precipitation, adsorption, or conversion (Shen et al., 2011). The class of microorganisms known as phosphate solubilization microorganisms (PSM) includes those that dissolve both organic and inorganic phosphates. These bacteria mineralize and solubilize insoluble organic and insoluble inorganic phosphorus (Granada et al., 2018). The formation of siderophores, organic and inorganic acids, hydroxyl ions, protons, and CO2 by microorganisms is one of the mechanisms by which inorganic phosphate is solubilized. These acids chelate cations or lower pH in order to release P. Phospholipases, phosphatases, and other extracellular enzymes are produced during the mineralization of organic phosphate (Richardson and simpson, 2011).

Bacillus spp. are among the most potent PSM, and plant/soil inoculation with them is a promising method for improving plant P absorption. According to Saeid et al, 2018, *Bacillus (B. subtilis, B. megaterium, and B. cereus)* solubilizing exudates are made up of gluconic, lactic, acetic, and succinic acids, demonstrating a high link between the overall concentrations of organic acids and the amounts of released phosphorus. The seed germination and growth characteristics of pepper, tomato and eggplant were positively affected by isolates of *, B. subtilis, B. megaterium and B. simplex*, which also produced propionic,acetic, isocaproic, caproic isobutyric, , and heptanoic acids (Bahadir et al., 2018).

Table 2.3: Mechanisms used by *Bacillus* to enhance plant growth

***Bacillus* species**	**Treated plants**	**Mechanism**	**Effects**	**Reference**
Bacillus amyloliquefaciens	Rice	ABA production	Increase in growth and stress tolerance capabilities	Shahzad et al., 2017
Bacillus subtilis	Tomato	ACC deaminase, IAA production	Increase in root and shoot biomass and chlorophyll contents	Xu et al., 2014
Bacillus spp.	Soybean	Production of ABA, CKs, IAA, Gas	Better growth and higher proline contents	Naz et al., 2009
Bacillus megaterium	Common bean	CKs production	Promotion of seedling growth	Ortiz et al., 2008
Bacillus subtilis	Lettuce	CKs production	Increase in root, shoot weight, and higher levels of CKs	Arkhipova et al., 2005
Bacillus subtilis	Tomato	GA and IAA production	Better root and shoot growth, enhanced hormone production, Increase in leaf area and seedling vigor	Chowdappa et al., 2015
Bacillus subtilis	Cucumber	Phosphate solubilization	Increase in plant growth, P uptake, and accumulation of Phosphorus	Garcia et al., 2016
B. simplex, B. subtilis, B. megaterium,	Tomato, pepper, eggplant	P-solubilization	Enhanced plant growth and seed germination	Bahadir et al., 2018

Bacillus sp.	Maize	Nitrogen (N_2) fixation	Increase in root volume and seed germination	Szilagyi et al., 2014

2.6.2. Phytohormones of *Bacillus*

By promoting the production of PGRs or phytohormones such auxins (IAA), ethylene, gibberellins, cytokinins, and abscisic acid, *Bacillus* spp. might directly boost plant yield. Organic compounds known as plant hormones have a very small but significant effect on a plant's physiology and growth. Different plants' subsequent growth enhancement has been directly linked to *Bacillus* spp.'s production of plant hormones (Miljaković et al., 2020).

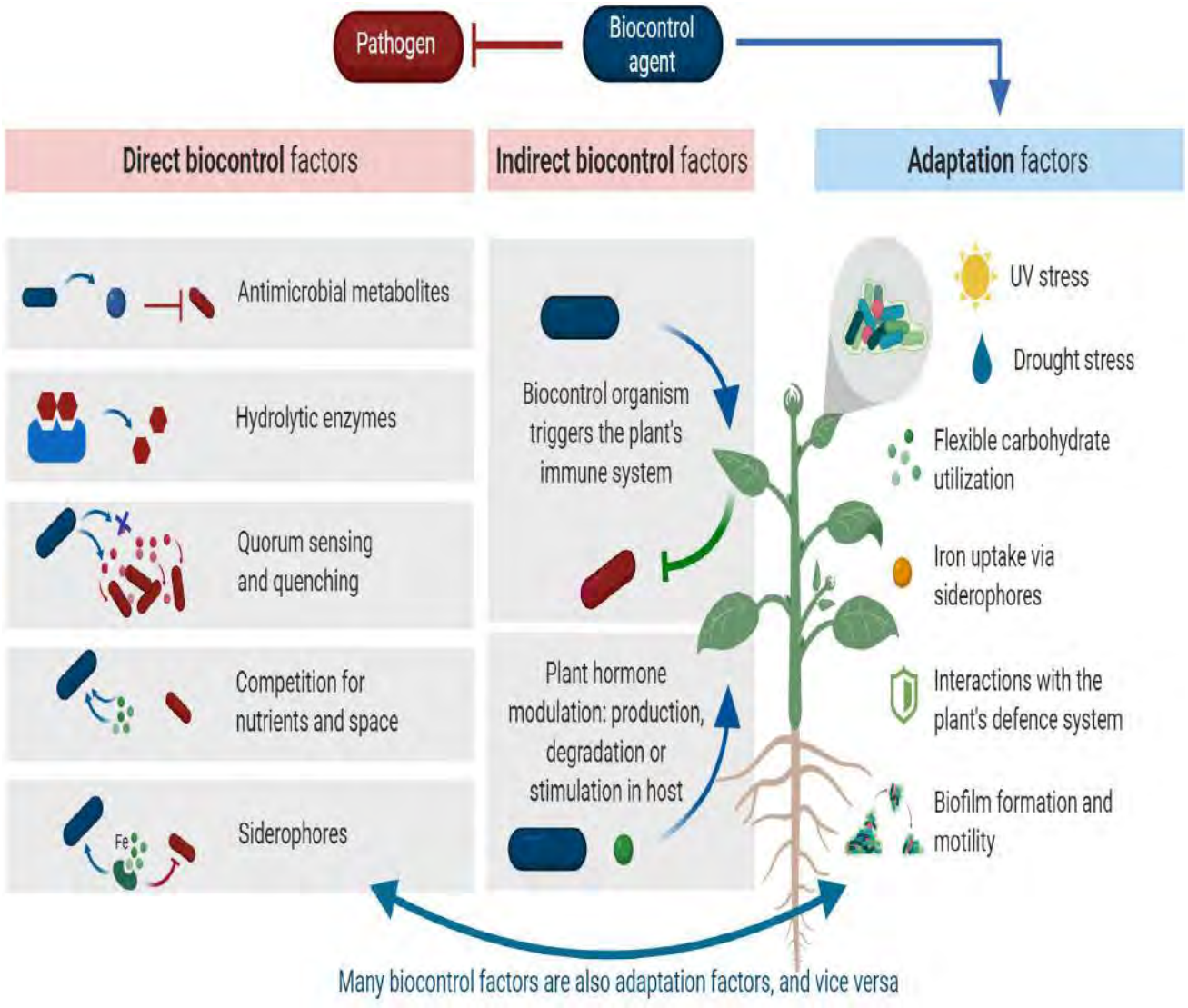


Figure 2.1: An overview of the biocontrol methods. Successful biocontrol agents have both biocontrol and adaptive features. Direct and indirect interactions between biocontrol agents can be distinguished. Direct interactions occur when a disease (red rod) and a biocontrol agent (blue rod) interact directly. The host plant's immunity against pathogen infection is boosted as a result of indirect interactions between the biocontrol agent and the host plant. The term "adaptation factors" refers to variables that must be taken into account in order to adjust to particular phyllosphere conditions, such as high UV stress levels, a lack of water and nutrients, and immune system reactions. Last but not least, biocontrol and adaptive elements frequently coexist (Legein et al., 2020).

2.7. Biosurfactants

Microbial surfactants constitute a group of amphipathic molecules having different constituents from different sources which have been explored for their properties to reduce surface tension. Their unique and extraordinary properties have ensured their applications in various fields of life e.g., healthcare, agriculture, medicine, environmental science and industries etc. These biomolecules are specific chemical compounds having a variety of chemical structures and are produced by yeasts, fungi and bacteria. They are unique amphiphilic compounds having both the hydrophobic (unsaturated or saturated fatty acids) and hydrophilic (peptides or amino acids, cations, polysaccharides ad anions etc.) moieties. The surfactin molecules of *Bacillus subtilis*, sophorolipids of *Candida bombicola*, emulsan of *Acinetobacter calcoaceticus*are Rhamnolipids of the*Pseudomonasaeruginosa*,are the various recognized biosurfactants. They lower the interfacial as well as surface tension between interfaces and molecules respectively and they are blessed with even more biological activities (hemolytic, antiviral, insecticide and antibacterial etc (Markande *et al*., 2021).

2.7.1. Lipopeptides

These are the active compounds of the microbial surface which are produced by an extensive range of fungi, yeasts and bacteria. The have extreme structural diversity and also possess the capability to lessen the interfacial and surface tension at the interface and surface respectively. Iturin, Surfactin, and fengycin molecules produced by the *Bacillus subtilis* are included in the most popular types of lipopeptides (Markande et al., 2021). Their ability to destabilize the biological membranes and form pores enable them to be used as antiviral, antimicrobial, antitumor as well as insecticide agents. The lipopeptides may also functionby acting at specific

surfaces and modulating the activity of enzymes. They may also cause the inhibition of some specific enzymes allowing them to be used as antifungal agents (Hamley, 2015).

2.7.2. *Bacillus*-Related Lipopeptides

Members of the *Bacillus* family secrete an array of antimicrobial and antifungalcompounds. Some of these compounds have ribosomal origin (subtilin, sublancin, subtilosin A and TasA), and the otherssuch as difficidin, chlorotetain, mycobacillin, rhizocticins, bacilysin, bacillaene,and the lipopeptides belong tothe iturin, fengycin and surfactin,families, are secreted bynon-ribosomal peptide synthetases or by polyketide synthases. The non-ribosomal peptide synthetases causes an excellent heterogeneity among the lipopeptides molecules which are generated by *Bacillus* with respect to the type andsequence of their amino acid residues, nature of their peptide cyclization, and the nature, length and branching of the fatty acid chains. Lipopeptides are grouped into three distinct families on the basis of their sequence of amino acids; fengycins, iturins, and surfactins (Jacques, 2011).

2.7.2.1. Surfactin

Surfactin is a lipoheptapeptide having cyclic nature and containsabout 7 residues of the L- and D-amino acids with an additional1 residue of a specific β-hydroxy fatty acid and their amino acid sequence is entirely different from the iturins. Diversity of peptide chainallowsthe distinction between lichenysin, surfactin, pumilacidin and esperin, in the surfactin family. It bears the capacity to decreasewater surface tension from original 72 to the less 27 mN/m at an effective concentration of as less as 0.005%. So, because of this extraordinary surfactant activity, it is named as a surfactin. The surfactins molecules are strong biosurfactants, showing antibacterial activity but notsufficient fungitoxicity (some exceptions are present).The recent studies have shown that the surfactin also exhibit potent anti-micoplasma, anticoagulant, antiviral and antitumoral, activities and they can also act as enzyme inhibitors (Mnif and Ghribi, 2015).

2.7.2.2. Iturin

Iturins-a class of CLPs have a peptide chain and a β-amino fatty acid attached by the amide bonds to their amino acid residues. They possess a mutual sequence (β-hydroxy fatty acid-Asx-

Tyr-Asx) and have shown variations at the other four positions also.The Iturin A, D, C, and E,bacillo-peptin,bacillomycinF, D, and L, and the myco-subtilins are members of iturin group. They represent a specific class of the pore-producing lipopeptidesand they also are well known for antifungal properties for a wide array of fungi and yeasts but their antibacterial functions are limited only to some bacteria e.g., *Micrococcus luteus*. In fact, this capability of the iturins to enhance the permeability of membrane cells is because ofdevelopment of the ion-conducting pores. The nature of these pores depends both on structure of peptide cycle and the lipid composition of membrane. Hence, their antifungal activity is due to strong interactions among the cytoplasmic membrane of their target cellswith iturinse.g., K^+ permeability is greatly enhanced for them (Ongena and Jacques, 2008).

2.7.2.3. Fengycin

Fengycin are a group oflipo-peptidespossessing10 amino acids and also a molecule of lipid which attached to their *N*-terminals. They differ are different from surfactin anditurin dueto the presence of some unusual amino acidse.g.,allo-threonine and ornithine. The diversified nature of peptide moiety (variants having a characteristic Ala-Val dimorphy at the position 6 in peptide ring) allows to classify fengycin family into Plipastatin A and B and Fengycin. Similar to the Iturins, the Fengycin shows antifungal properties and cause inhibition of growth of an extensive range of phytopathogens such as filamentous fungi (Moyne. 2001).

Table 2.4: Commercially available preparations of *Bacillus* as biocontrol agent (Miljaković et al., 2020)

***Bacillus* species**	**Plants**	**Preparation**	**Company**
Bacillus spp.	Various crops	*Bacillus* SPP®	Bio Insumos Nativa, Chile
Bacillus sp.	Various crops	Sublic®	ELEP Biotechnologies, Italy
Bacillus velezensis	Fruits, vegetables	Botrybel®	Agricaldes, Spain
Bacillus licheniformis	Various crops	EcoGuard®	Novozymes A/S Denmark, Novozymes Biologicals, USA
Bacillus pumilus	Fruits, vegetables	Sonata®	AgraQuest Inc., USA
Bacillus pumilus	Cotton, sugar beet, cereals, vegetables, legumes,	Yield Shield®	Bayer CropScience, USA
Bacillus amyloliquefaciens	Ornamentals,cereals, vegetables,	RhizoVital®42, RhizoVital®42TB	ABiTEP GmbH, Germany
Bacillus amyloliquefaciens	Tobacco, vegetables, legumes,	BioYield®	Gustafson Inc., USA
Bacillus subtilis	Legumes, fruits, vegetables, and other plants	Ecoshot®	Kumiai Chemical Industry, Japan
Bacillus subtilis	Cotton, vegetables, legumes	Bio Safe®	Lab. Biocontrole Farroupilha, Brazil
Bacillus subtilis	Ornamentals, soybean, and others	Pro-Mix®	Premier Horticulture Inc., Canada
Bacillus subtilis	Cotton, legumes, vegetables, and other plants	Subtilex®	Becker Underwood, Inc., USA
Bacillus subtilis	Maize, vegetables, legumes	Companion®	Growth Products Ltd., USA

3. Materials & methods

3.1. Sample collection

Agriculture soil was collected from various strawberry fields of Charsada, Swat. Samples from different fields and from different areas of same field were collected using sterile spatula. The soil was dug 6cm deep and samples were placed in sterile zipper bags and taken to the Environmental and Applied Microbiology lab at Quaid-i-Azam University, Islamabad for further processing. Soil samples from rhizospheric areas of strawberry plants were collected and taken to Microbiology lab of QAU till August 2022.

3.2. Sample enrichment

Culture enrichment technique was employed for further processing of soil samples. Strainer was used to make soil free of stones and gravel. 1g of soil sample was added to 100ml of autoclaved Minimal Salt Media (MSM) (Table 3.1) in Erlenmeter's flask. Flasks were left for incubation for about 1 week in shaking incubator at 37C and at 150rpm.

Table 3.1: Composition of Minimal Salt Media (MSM) (a)

Macronutrients	Concentration g/L
K_2HPO_4	10
$NaH_2PO_4.2H_2O$	5
$NaNO_3$	2
$CaCl_2.2H_2O$	0.01
$MgSO_4.7H_2O$	0.2
$FeSo_4.7H_2O$	0.08
Glucose	20

Table 3.2: Composition of Minimal Salt Media (MSM) (b)

Micronutrients	Concentration mg/L
$MnSO_4.4H_2O$	0.80
$CoCl_2.6H_2O$	1.20
$CuSO_4.5H_2O$	1.20
$ZnSO_4.7H_2O$	1.40

3.3. Isolation of *Bacillus* strains

Two methods were employed for the isolation of *Bacillus* strains i.e., direct sprinkle method and Serial dilution method. In direct sprinkle method soil sample was directly sprinkled on plate containing Hi-Crome *Bacillus* agar. Commercially available differential Hi-crome media is specifically used for the isolation of *Bacillus* species in mixed culture by chromogenic method. Plates were incubated at 37°C for 48hrs.

In serial dilution, 27 autoclaved tubes were used i.e., 9 tubes for each sample. Dilution of 0.9g/100ml was made by adding 1g of enriched culture sample in 9ml of normal saline. Each sample was then subjected to serial dilution by transferring 1ml of the sample from 1st tube to subsequent tubes containing 9ml of saline water. The same method was followed till 9th test tube of the last stack i.e. 27th one.

From each diluation set out of 3, 500µl was taken from diluation no. 3, 5 and 7 and was spread onto Hi-crome *Bacillus* agar plate using sterile spreader. Plates were left for incubation at 37°C for 48hrs.

3.4. Purification of *Bacillus* strains

After incubation dense growth of *Bacillus* strains was observed on plates. On the basis of different morphological characteristics such as color, growth pattern, pigmentation and texture of various isolates were sub-cultures on autoclaved Nutrient Agar plates. This process keeps on repeating until pure isolates/colonies are obtained. Further morphological and biochemical examination of various isolates were carried out.

Table 3.3: Chemical Ingredients of Hi chrome *Bacillus* Agar Media

Compositions	Concentration g/l
Agar	15
Peptic digest of animal tissue	10
Meat extract	1.0
Sodium Chloride	10
Chromogenic mixture	3.2
D-Mannitol	10
Phenol Red	0.025

3.5. *S*econdary screening of *Bacillus* strains having antifungal potential

Spore suspension of 7 days old fungal culture was prepared by taking spores of pathogenic fungus in 1ml normal saline using McFarland as a standard. Spore suspension of each pathogen was spread on SDA plate while bacterial culture was point inoculated at the center. Plates were left for incubation at 37℃ and results were observed after 24, 48, 72, 96 and 120hrs.

3.5.1. MacFarland turbidity standard preparation

For the preparation of 0.5 MaFarland standard 1.175% w/v of $BaCl_2$ and 1% v/v H_2SO_4 was used. It was prepared by adding 85ml of 1% H_2SO_4 and 0.5ml of 1.175% $BaCl_2$ drop by drop while constantly swirling the flask. Bring volume to 100ml by addition of 1% H_2SO_4 , homogenize the mixture by placing it on a magnetic stirrer for 3-5 mins. Check OD at wavelength 625nm. Maintain the OD at 0.08-0.10. Put cap on tubes tightly and seal it with parafilm and store it in dark at room temperature for about 3-4 months.

3.5.2. Normal saline preparation

For the preparation of 0.9% w/v of the normal saline solution, add 0.9g of NaCl in 100ml of distilled water.

3.6. Isolation of fungal phytopathogens

Fungal pathogenic strains were isolated from diseased areas of root, stem and leaves of strawberry plants. Mixed culture of fungal pathogens was obtained initially. Then it was subjected to sub-culturing on the basis of different morphological characteristics i.e. color and texture etc. Fungal cultures were sub-cultured and refreshed by growing them on Sabouraud Dextrose Agar (SDA) plates at 28℃. This process keeps on repeating until pure fungal cultures are obtained in each plate.

3.7. Identification of selected *Bacillus* isolates

3.7.1. Microscopic analysis

Microscopic analysis of selected strains was performed by making their smears on a clean glass slide. Gram staining was performed following basic staining protocol for bacterial strains. Slides were observed under microscope at 100X lens.

3.7.2. Biochemical identification of selected *Bacillus* isolates

Various biochemical assays were performed for the selected strains (MQ1-MQ8).

3.7.2.1. Catalase assay

Production of Catalse enzyme was evaluated by picking a single colony from one day old culture and placing it on a glass slide. Bubble formation was observed after flooding glass slide with 1% H_2O_2.

3.7.2.2. Oxidase assay

For detection of enzyme Cytochrome Oxidase, small size filter paper disc was soaked in 1% Kovac's reagent. After drying one day old culture colony was rubbed onto filter paper disc and the color change was observed.

3.7.2.3. Citrate test

To check utilization of Citrate as a sole C-source, Simmon citrate agar slants were prepared in test tubes. Surface streaking was done on slants and was left for incubation at 37℃ for 24hrs. Color changes were observed after incubation.

3.7.2.4. Indole utilization test

The ability of selected strains to degrade amino acid trptophan was evaluated by Indole test. Tryptophan broth inoculated with selected strains was incubated for 24hrs at 37℃. Color transformation was detected by adding 5 drops of Kovac's reagent (p.dimethylamino benzaldehyde).

3.7.2.5. Urease test

2.95g of urea powder was dissolved in 150ml of distilled water to make a urea broth. After autoclaving the media, urea was added to avoid initial urea decomposition. Utilizing a wire loop, aseptically inoculate the selcted organism sample. Tubes were incubated for 24 hours, at 37 °C.

3.8. Preservation of selected isolates

Subculturing was used to obtain pure cultures for preservation. A loop full of culture was inoculated in nutrient broth and cultured in a shaking incubator at 37°C for 24 hours. 500 microliters of bacterial broth and 1 milliliter of 30% oven-heated glycerol were poured in an eppendroff and kept at -20˚C for a long period.

3.9. Detection of hydrolytic enzymes of *Bacillus*

Following enzyme assays were performed to evaluate the production of hydrolytic enzymes by selected *Bacillus* strains.

3.9.1. Protease assay

Qualitative protease assay was done on Modified Basal Media (MM). Strains were inoculated at the center and plates were incubated for 24hrs at 37℃. After incubation, plates were flooded with 70% acetic acid and zone of hydrolysis were evaluated. Composition of MM is given in table 3.4.

Table 3.4: Composition of Modified basal media

Media Composition	Quantity (g/L)
Glucose	1
Yeast	2.5
Agar	14
Skim milk protein/	6.2
Or Casein	5

3.9.2. Endoglucanase assay

Cellulase producing ability of Bacterial strains was evaluated by this assay. Pure culture was inoculated at the center pre-poured with 1% carboxymethyl cellulose and 1M NaCl.. After 24hrs incubation at 37℃, plates are flooded with 0.1% Congo red and zone of hydrolysis was observed.

3.9.3. Amylase assay (starch iodine test)

Isolated colonies were picked and are inoculated at the center of plates containing starch as a carbon source. Plates were left for incubation at 37℃ for 24hrs. Clear zone of hydrolysis could be observed after placing few iodine crystals onto the cap of Petri dish and placed plates upside down for few minutes.

Table 3.5: Composition of starch media

Media Composition	Quantity (g/L)
Starch	2
Peptone	5
Beef extract	3
Agar	15
Distilled water	1000ml

3.10. Antifungal properties of *Bacillus* strains by dual culture assay

3.10.1. Point inoculation method

Normal saline and fungal spores were used to make a fungal spore suspension. The turbidity of pathogen suspension was compared to a 0.5 percent McFarland turbidity standard. To spread spore suspension over SDA plates, sterile cotton swabs were utilized. The center of the plate was inoculated with a loop of Bacterial culture. Plates were incubated for 72 hours at 30°C in a fungal incubator.

3.10.2. Agar well diffusion method

The *Bacillus* strains were cultivated in 200 mL nutrient broth for 48 hours in a shaking incubator at 37°C. Centrifugation at 10,000 rpm for 20 minutes yielded cell-free supernatant. Using a sterile borer, 6mm diameter wells were created in SDA plates, and wells were sealed from the base with one drop of agar. Fungal pathogens were loaned on SDA plates using sterile cotton swabs. Wells were filled with 100 microliters of bacterial supernatant and incubated for 72 hours at 30˚C. Around the wells, inhibitory zones were measured.

3.10.3. Inhibition percentage (%) by dual culture assay

Petri plates with SDA media were prepared, and the bacteria was inoculated by line streaking 2 cm away from the fungal plug using the dual assay described by (Limtong et al., 2020) with slight modification. A 5 centimeter plug was inoculated in the centre. As a control, a plate with simply a fungus' mycelial plug was used. Plates were incubated for five to seven days at 27.2°C. Replication was applied to every treatment. Formula for percent growth inhibition is given below.

Growth Inhibition (%) = (Radius of fungus cultured alone—Radius of fungus cultured with bacteria)/Radius of fungus cultured alone * 100

3.11. Screening for Plant Growth Promoting Traits of *Bacillus*

3.11.1. Ability to Solubilize Phosphate

NBRIP medium with 5% of tri-calcium phosphate was used to examine the ability of the selected bacterial strains to solubilize P. Bacterial strains were spot-inoculated on NBRIP agar medium

and incubated at 30 °C for 72 hours. The Phosphate Solubilization Index was used to determine the efficiency of phosphate solubilization.

Table 3.6: Composition of NBRIP agar media.

Composition	Conc./ 1000mL
Glucose	10g
$MgSO_4.7H_2O$	0.25g
$MgCl_2.6H_2O$	5g
KCL	0.2g
$(NH_4)_2SO_4$	0.1
$Ca_3(PO_4)_2$	5g
Agar	15%

3.11.2. Indole Acetic Acid (IAA) Production Test:

Qualitative assessment of IAA production was checked by inoculating selected bacterial strains in 30 mL of NBRIP broth which is supplemented with tryptophan and slowly shacked at 28°C for 3 to 5 days. After incubation step, broth was centrifuged at 10,000rpm at 4˚C for 10min. 2 mL of Salkowski reagent (35% $HClO_4$ and 0.5M $FeCl_3$) was mixed with 2 mL of cell-free supernatant. Then the mixture was kept at room temperature in dark for 20 to 30min. IAA production is indicated by the appearance of pink-red color.

3.11.3. HCN Production Test:

The top of the plate was covered with a Whatmann No. 1 filter paper socked in a solution of 2% sodium carbonate and 0.5% picric acid. Parafilm was used to seal the plates, which were then incubated for 4 days at 28 2°C. HCN production was indicated by the orange/red color.

3.11.4. Biofilm formation

The Congo Red Assay was demonstrated by Freeman et al. in 1989 to determine the generation of biofilms. The chosen bacteria were grown on Congo red agar, a mixture of BHI agar, sucrose, and Congo red dye. At 37°C, an incubation time of 24 to 48 hours was recorded. *Bacillus* species with red colonies are powerful biofilm producers.

3.12. Extraction of lipopetides

Using the solvent extraction technique, crude lipopeptide was obtained from selected *Bacillus* strains using ethyl acetate. For lipopeptide extraction, 600 mL MSM was prepared. 24 hours fresh bacterial culture was inoculated in MSM broth and kept in a shaking incubator at 37°C for 72 hours. Cell-free supernatant was collected after 3 days of incubation by centrifuging MSM broth at 10,000 rpm for 20 minutes at 4˚C. The pH of the supernatant was kept at 2 with HCL and incubation was done overnight at 4˚C. Following that, 600 mL of ethyl acetate was added to 600 mL of supernatant and thoroughly mixed. Place in separating funnel for 2 hours after completely mixing to separate two layers. The upper layer of solvent with the compound was recovered and the lower layer of supernatant was discarded. After using rotary lipopeptide was collected and ethyl acetate was recovered, and allowed to air-dry overnight.

3.12.1. Characterization of lipopetides produced by selected *Bacillus* strains

The General Microbiology Lab 2 of Quaid-i-Azam University Islamabad conducted an FT-IR analysis of extracellular crude lipopeptide. Tensor 27, an FT-IR spectrophotometer from Bruker Germany, was used in this study. Solvent extraction was used to extract crude lipopeptide, and measurements were taken in the transmittance mode between 400 and 4000nm. The chemical bonds of the test sample were determined using this technique, which gives the idea of functional groups of the test sample based on the absorption of specified wavelengths.

3.13. DNA extraction of selected *Bacillus* strains MQ7 & MQ8 using Phenol-Chloroform-Isoamyl (PCI) method

Extraction of DNA was done for identification and molecular analysis of isolated bacterial strains. DNA was extracted using the PCI method.

1. A loop containing 24 hours of fresh bacterial culture was inoculated in Nutrient Broth and incubated for 24 hours at 37°C.2 mL of bacterial broth was centrifuged at 10,000rpm for 20 minutes at 4°C. The supernatant with no cells was discarded, and the pallet was collected.
2. To disrupt the cell wall of bacteria, 30-50 µL of 10% SDS (Sodium Dodecyl Sulfate) was applied.

3. 5 µL of Proteinase K was added for protein digestion by generating peptide bond breaking, and the mixture was placed in a water bath at 37°C for 1 hour. Proteinase K aids in the release of nucleic acids and causes DNAse and RNAse inactivation.
4. For the elimination of DNA binding protein, 80 µL of 5M NaCl was added. It aids in the retention of proteins in the aqueous layer and the prevention of DNA precipitation in alcohol.
5. Then, for polysaccharide separation, 100 µL of CTAB (Cetyl Trimethyl Ammonium Bromide) buffer a cationic detergent was added and placed in a water bath at 65°C for 10 minutes.
6. For separation of the organic and aqueous layers, 500 µL of Phenol Chloroform Isoamyl (PCI) in a 25:24:1 ratio was added.
7. The sample was centrifuged at 10,000 rpm for 15 minutes at 4°C.
8. The upper layer was removed and transferred to a new eppendrof. 500 µL PCI was added again and centrifuged at 10,000 rpm for 15 minutes at 4°C.
9. The upper layer was again removed and transferred to a new eppendrof. 500 µL of isopropanol and 300 µL of sodium acetate was added and incubated at room temperature for 30 minutes. For precipitation, the sample was held at 4°C overnight. The next day after centrifuging the sample at 8,000 rpm for 6 minutes at 4°C, a pellet was obtained.
10. The next step is the washing step, which involves adding 200 µL of 70% ethanol and centrifuging for 2 minutes. Low temperature protects DNA from DNAse action, hence cold ethanol is preferred.
11. To dissolve the pellet, ethanol was discarded and 50-100 µL of TE buffer was added..
12. The samples were then kept at -20°C.

3.13.1. Gel electrophoresis for DNA detection

To make an agarose gel (1X), 27 mL of distilled water was mixed with 0.3g of agarose and 3 mL 10X TBE (Tris Boric acid EDTA) buffer. The gel was heated in the oven for 2 seconds time intervals for making a transparent solution. The agarose gel was allowed to cool for a few

minutes before adding 3 µL of ethidium bromide (Et. Br). A comb of the appropriate size was put in the gel after the solution was poured into the gel tray. After solidification, the gel was put in a 1X TBE buffer electrophoresis chamber. 3 µL of DNA sample was combined with 3 µL of loading dye and placed into Agarose gel wells that become solidified. The voltage, current, and time were all set to 110 volts, 500 milliamps, and 30 minutes. After that, the gel was examined for DNA bands using a UV transilluminator at low and high resolution. To adequately examine DNA presence and quality, photographs were taken with the assistance of a Digital Camera DC 290 (Kodak, New York, USA). After acquiring DNA samples of suitable size and quality, the samples were sent to be sequenced using the 16S rRNA gene for bacterial strain identification.

3.14. Polymerase Chain Reaction (PCR) of lipopeptide biosynthetic genes (Fengycin & iturin)

PCR was used to find the biosynthesis genes of lipopeptides (Fengycin and iturin). Primers were used on NRPs producing *Bacillus* strains.

3.14.1. Working solution of primers

The primers were diluted according to the dilution methodology listed on the package. The working solution is made by diluting it with PCR water at a 9:1 ratio.

3.14.2. Reaction mixture for PCR

20 µL of the reaction mixture was prepared. Master from WizPure^{TM} PCR 2X Master.

3.14.3. PCR amplification

For each set of primers, reaction conditions were already optimized by running annealing temperature gradient in gradient thermocycler PCR of (Bio-Rad) company. For 35 cycles of PCR thermal cycle program includes initial denaturation temperature at 94℃ for 2 minutes, followed by a denaturation step at 94℃ for 1 minute. The annealing temperature for Fengycin and Iturin gene was set at 45℃. Extension for each set of primers was done at 72℃ for 45 seconds, followed by a final extension step of 72℃ for 10 minutes.

3.14.4. Gel electrophoresis of PCR products

To make a 2X Agarose gel, 0.6g Agarose, 3 mL 10X TBE buffer, and 27 mL distal water was used. For making the transparent solution, the gel was heated in the oven for 2 second time

intervals. The agarose gel was allowed to cool for a few minutes before adding 3 µL of ethidium bromide (Et. Br). After solidification, the gel was placed in an electrophoresis chamber containing 1X TBE buffer.3 µL of the amplified PCR product was loaded into wells, along with 3 µL of loading dye. GenOn provided commercially available loading dye. For 30 minutes, the gel was run at 110V. In a UV transilluminator, bands of related genes were observed (Weal Tax). A gene ladder of 1kb was utilized to assess band size. Solis BioDyne provided us with a 1kb DNA ladder.

3.15. Greenhouse experiment for anti-fungal and PGPR activity of selected *Bacillus* strains

A field experiment was conducted in triplicates in the summer season between June 2023-August 2023 in a greenhouse at QAU University Islamabad, to assess the biocontrol and plant growth promoting activity of selected bacterial strains. An agriculture soil from NARC was used which has the following chemical properties. pH of the soil was 7.25, Electrical conductivity 4.25 dS/m, Organic matter 3%, Available phosphorus 28.6 mg/kg, , Saturation 40%. *Zea maize* seeds were used for biocontrol activity. Seeds were surface sterilized with hypochlorite bleach for 1 minute, then rinsed with 95% ethanol for 1 minute, and finally washed three times with distal water. Control seeds were soaked in distal water and respective seeds in appropriate broth culture and supernatant for 3 hours before sowing. The soil was separated into two halves, with one of them being autoclaved for 40 minutes at 121°C (AS). Normal soil was defined as soil that had not been autoclaved (NS).

3.15.1. Preparation of seedling

Seedling pots were filled with both normal and autoclaved soil for biocontrol activity.

3.15.2. Inoculum preparation

In a 250 mL Erlenmeyer flask, biocontrol bacterial strains were inoculated in MSM and incubated for 72 hours at 37°C. After centrifugation at 10,000 rpm for 15 minutes at 4°C, the supernatant was collected. In addition, strains were inoculated in nutrient broth and incubated for 24 hours at 37°C.

3.15.3. Fungal test pathogen

In a 250 mL Erlenmeyer flask, SDB was made and inoculated with a fungal test pathogen. For one week, pathogen cultures were cultured at 150 rpm and 30°C in a shaking incubator.

3.15.4. Application of inoculum

Each pot was inoculated with the calculated amount of biocontrol agent, supernatant, and pathogens.

- Bacterial culture= 5ml
- Supernatant=5ml
- Pathogen=2.5ml

3.15.5. Treatments

Table 3.7: Treatments for pot experiment on *Zea maize* plant (Each experiment was performed in triplicates)

Autoclaved soil (AS)Treatments	Un autoclaved soil (US) Treatments
T1= Sterilized seed (negative control)	T1= Sterilized seed (negative control)
T2= *Fusarium oxysporum(FO)*-positive control	T2= *Fusarium oxysporum(FO)*-positive control
T3= MQ7	T3= MQ7
T4=MQ8	T4=MQ8
T5=MQ7+FO	T5=MQ7+FO
T6=MQ8+FO	T6=MQ8+FO
T7=CFS-MQ7	T7=CFS-MQ7
T8=CFS-MQ8	T8=CFS-MQ8
T9=CFS-MQ7+FO	T9=CFS-MQ7+FO
T10=CFS-MQ8+FO	T10=CFS-MQ8+FO
T11=MQ7+MQ8	T11=MQ7+MQ8
T12= MQ7+MQ8+FO	T12= MQ7+MQ8+FO

T13=FO+commercial fungicide 1 (Pyranazole)	T13=FO+commercial fungicide 1(Pyranazole)
T14=FO+ commercial fungicide 2 (Fosetyl-aluminium)	T14=FO+ commercial fungicide 2 (Fosetyl-aluminium)

As a result of pilot scale experiment several parameters were measured in order to check the plant growth. These parameters include: Root + Shoot length (cm), Chlorophyll content (using SPAD 502 plus chlorophyll meter), wet mass (g), dry mass (g), no of leaves, seedling vigour index (SVI), disease incidence, disease control(%) and increase in plant growth (%).

3.16. Statistical analysis

Statistics 1.0 software was used to analyse the data and analysis of variance (ANOVA) was done. Means were compared by Tukey's pair wise comparison and means were considered significantly different by considering significance level of $p \leq 0.05$ (Limtong et al., 2020).

Seed germination (%) = (no of germinated seeds/total no of planted seeds) × 100

Disease control (%) = [(disease incidence of positive control − disease incidence of each treatment)/disease incidence in positive control] × 100

Disease incidence (%) = [(SVI of negative control − SVI of positive control or treatment)/SVI of negative control] × 100

Seedling vigor index (SVI) (%) = (stem length + root length) × seed germination (%)

4. Results

4.1. Isolation, screening and identification of *Bacillus* strains

Bacillus colonies of various morphologies formed on the spread plates after 24 hours. Size, forms, shapes, appearance, margin, optical property, texture, and elevation were all considered when selecting colonies. Colonies were streaked on pre-poured Hi-chrome agar and 34 distinct colonies were obtained as a result. On the basis of initial screening of strains for their anti-fungal activity, 8 potential strains were selected, including: MQ1, MQ2, MQ3, MQ4, MQ5, MQ6, MQ7 and MQ8.

Table 4.1: Cultural characteristics of selected *Bacillus* strains

Strain code	Shape	Margins	Elevations	Size	Appearance	Color	Texture
MQ1	Circular	Entire	Raised	Small	Glistening	Light green	Mucoid
MQ2	Circular	Entire	Flat	Small	Rough	Yellow	Mucoid
MQ3	Circular	Entire	Flat	Small	Glistering	Green	Mucoid
MQ4	Punctiform	Entire	Raised	Small	Glistering	Green	Mucoid
MQ5	Punctiform	Entire	Flat	Small	Rough	Light green	Mucoid
MQ6	Circular	Entire	Flat	Small	Rough	Light green	Mucoid
MQ7	Irregular	wavy	Raised	-	Rough	White	Mucoid
MQ8	Punctiform	Entire	Flat	Small	Glistering	Green	Mucoid

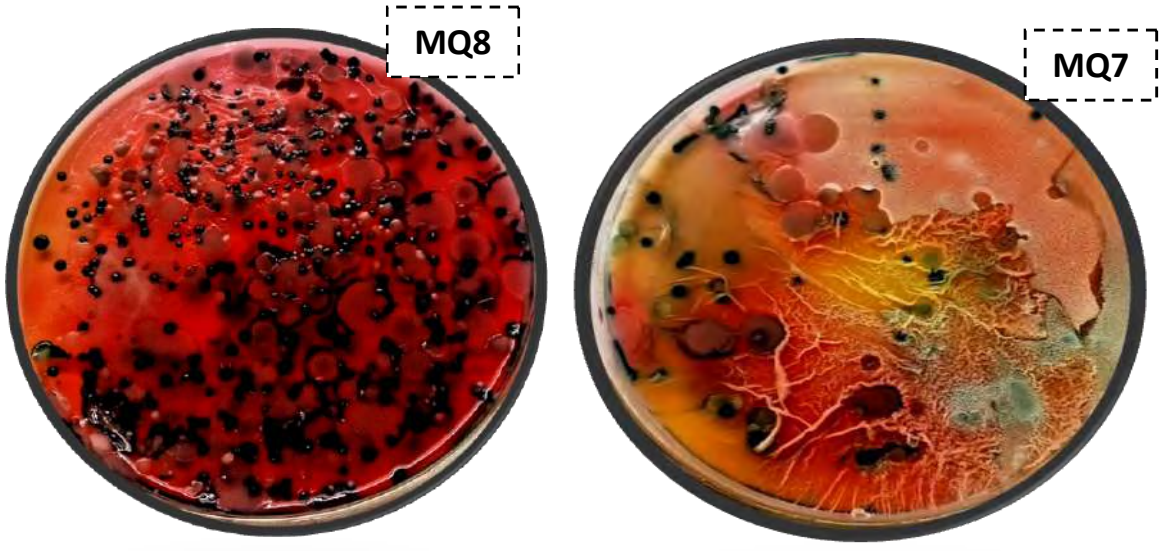
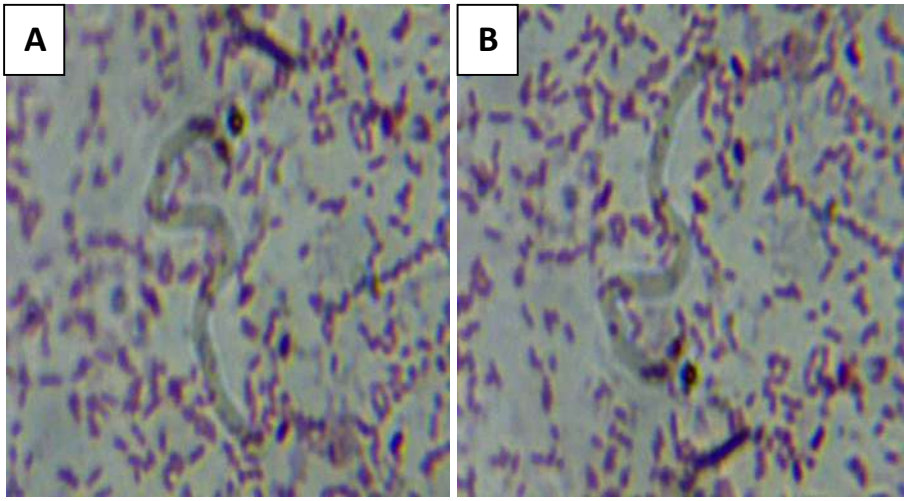


Figure 4.1: Growth pattern on Hi-Chrome agar media of selected *Bacillus* strains MQ7 and MQ8

4.2. Microscopy

Selected strains were viewed using a microscope (100X), and they appeared as gram-positive rods, as shown in images (Figure 4.2)



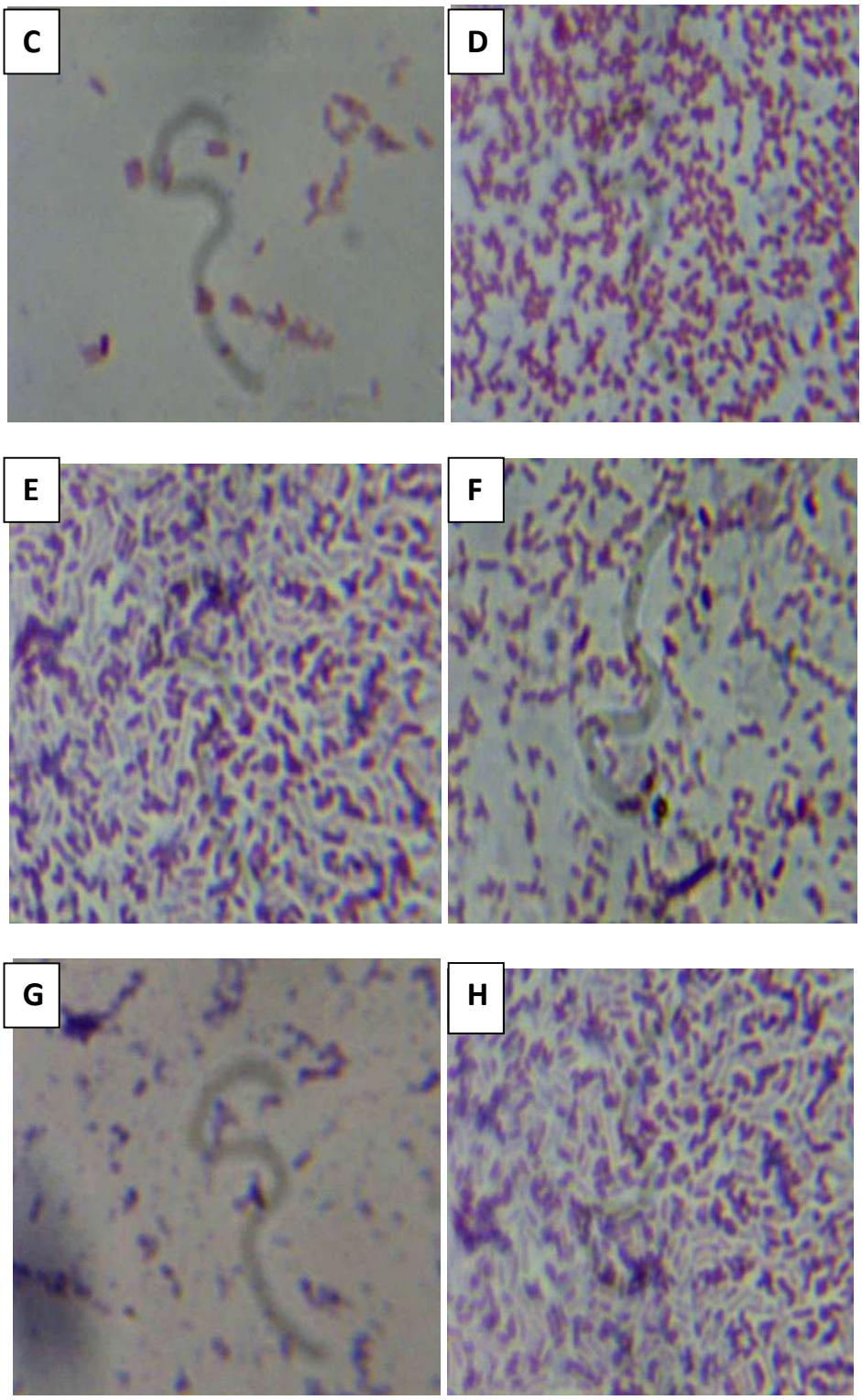


Figure 4.2: Microscopy of selected strains (100X)-A=MQ1, B=MQ2, C=MQ3, D=MQ4, E= MQ5, F= MQ6, G= MQ7, H=MQ8

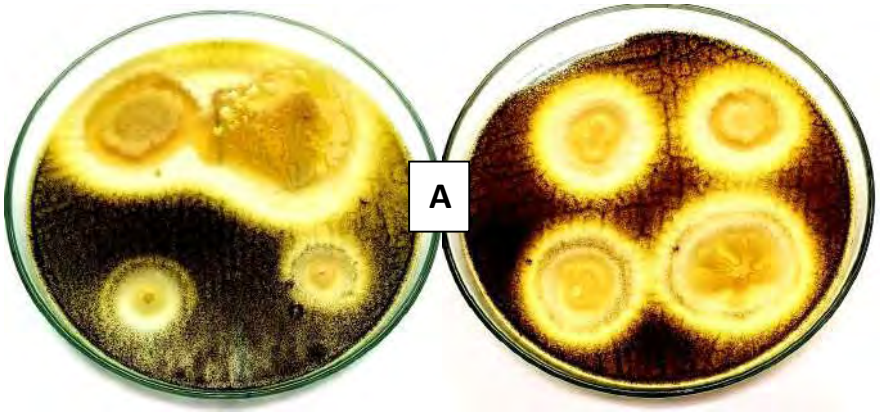
4.3. Biochemical tests

Table 4.2: Results of biochemical tests

Tests	MQ1	MQ2	MQ3	MQ4	MQ5	MQ6	MQ7	MQ8
Catalase	+	+	+	+	+	+	+	+
Oxidase	+	+	+	+	+	_	+	_
Citrate	+	+	+	+	+	+	+	+
Urease	-	-	-	-	-	-	-	-
Indole	+	+	+	+	+	+	+	+

4.4. Secondary screening of *Bacillus* strains

All of the 34 isolates were subjected to initial screening test via point inoculation assay. Based on zones of inhibition observed by cross-streaking fungal pathogens with *Bacillus* isolates, 8 strains with efficient anti-fungal activity were selected. The fungal strains were named as MQ1,/MQ2, MQ3, MQ4, MQ5, MQ6, MQ7, and MQ8.



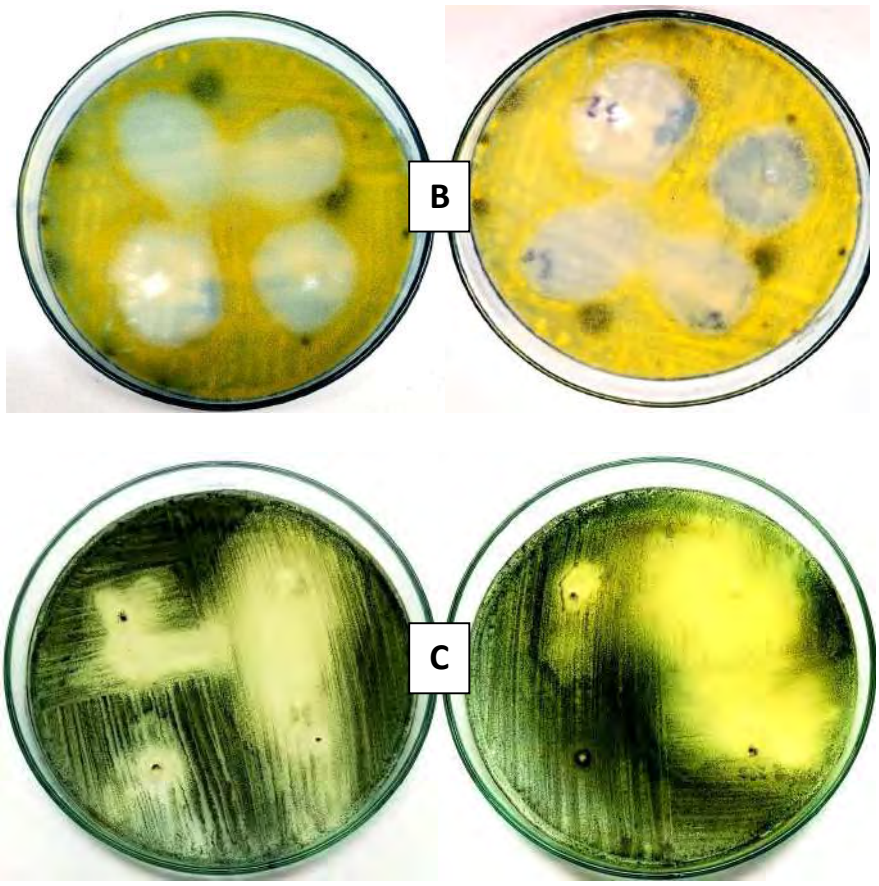


Figure 4.3: Initial screening on the basis of antagonistic activity of bacterial strains on SDA against fungal phytopathogens, A= *Aspergillus niger*, B= *Aspergillus flavus*, C= *Aspergillus fumigatus*

4.5. Antifungal properties of *Bacillus* strains against fungal phytopathogens by dual culture assay

To evaluate the antagonistic potential of *Bacillus* strains with various anti-fungal tests such as spot inoculation and agar well diffusion method by Cell Free Supernatant (CFS). Results of the mentioned experiments are discussed further.

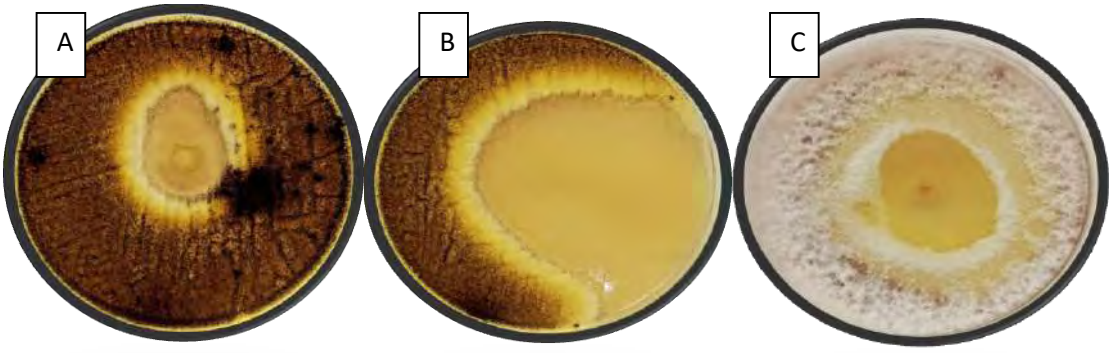
4.5.1. Spot inoculation method

Table 4.3: Zones of inhibition produced by selected *Bacillus* strains against fungal phytopathogens (Point inoculation)

Strain code	*A. flavus (mm)*	*A. niger (mm)*	*F. oxysporum (mm)*	*A. fumigates (mm)*
MQ1	25±5	32±2	16±4	-
MQ2	34±2	70±2	22±2	-
MQ3	22±5	56±2	40±0	-
MQ4	50±2	20±0	25±5	-
MQ5	25±0	45±0	20±5	55±2
MQ6	20±0	20±2	32±2	-
MQ7	55±2	56±5	23±2	15±5
MQ8	35±5	48±2	35±5	10±2

Each value represents mean, ± indicates Standard deviation (SD)

Inhibition of fungal mycelia by *Bacillus* strains was observed after 72hrs of incubation at 30C. Maximum zone of inhibition of 55mm was observed against *Aspergillus flavus*, 70mm against *Aspergillus niger*, 40mm against *Fusarium oxysporum* and 55mm against *Aspergillus fumigates*. A diverse range of antifungal activity was demonstrated by different *Bacillus* strains as depicted in table 4.3. Best selected pictures of inhibitory zones of fungi around centrally spotted bacterial strains are attached below.



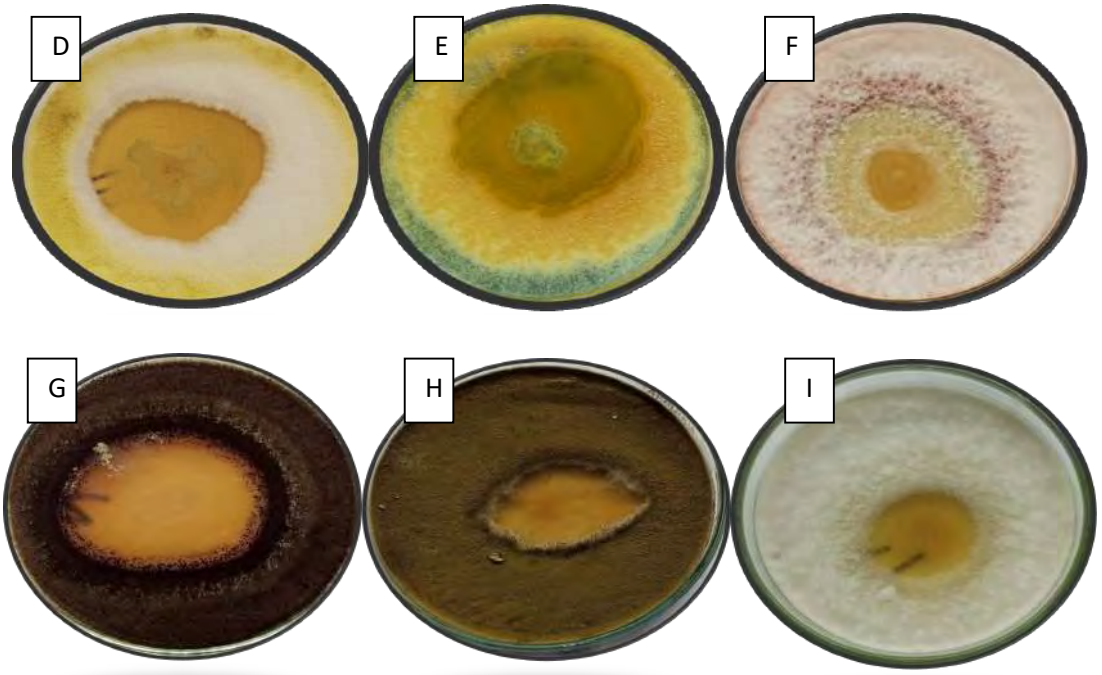


Figure 4.4: Zones of inhibition produced by selected *Bacillus* strains against fungal phytopathogens by spot inoculation method: A=MQ1 against *A. niger, B*=MQ2 against *A. niger*,C=MQ3 against *F. oxysporum*,D=MQ4 against A. *flavus*,E=MQ5 against *A.fumigatus* ,F=MQ6 against *F. oxysporum*,G=MQ7 against *A. niger* ,H=MQ8 against *A. flavus,* I= MQ8 against *F. oxysporum*

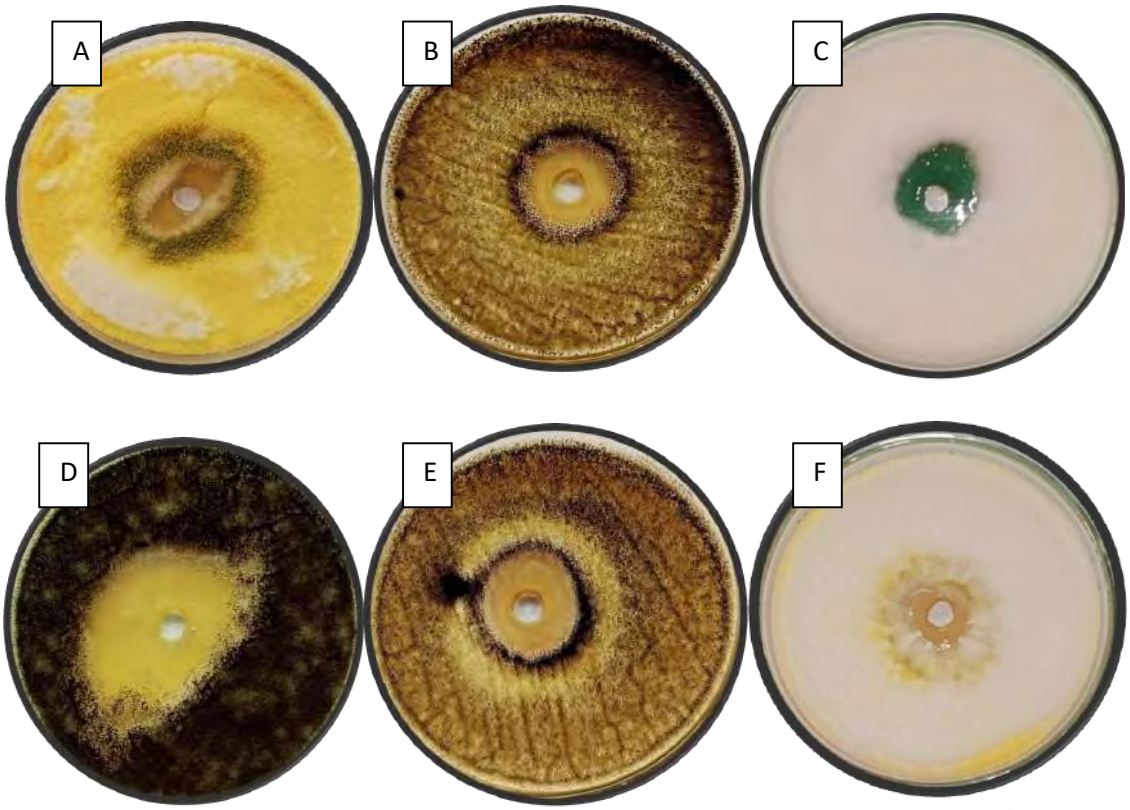
4.5.2. Agar well diffusion method

Table 4.4: Zones of inhibition produced by selected *Bacillus* strains against fungal phytopathogens

*S*train code	*A. flavus (mm)*	*A. niger (mm)*	*F. oxysporum (mm)*
MQ1	18±2	15±0	15±0
MQ2	30±2	35±0	25±5
MQ3	-	-	16±2
MQ4	35±5	-	-
MQ5	55±5	15±2	25±5
MQ6	50±4	52±3	30±0
MQ7	35±2	35±2	40±2
MQ8	25±0	30±5	50±2

Each value represents mean, ± indicates Standard deviation (SD)

Zone of inhibition was observed around the wells carrying supernatant. Maximum zone of inhibition of 55mm was observed against *Aspergillus flavus* by MQ5, 52mm against *Aspergillus niger* by MQ6, 40mm and 50mm against *Fusarium oxysporum* by MQ7 and MQ8 respectively. Cell Free Supernatants (CFS) of selected strains demonstrated a diverse range of antifungal activity against fungal pathogens depicted in Table 4.4. Best selected pictures of zones of inhibitions against fungi around wells carrying supernatants of *Bacillus* strains are shown in figure 4.5.



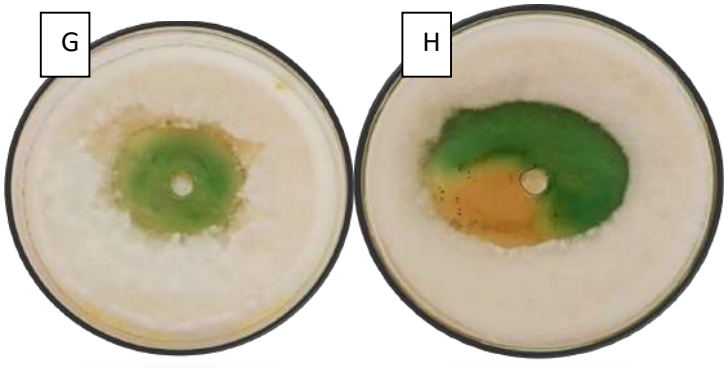


Figure 4.5: Zones of inhibition produced by selected *Bacillus* strains against fungal phytopathogens by well-diffusion method: A=MQ1 against *A. flavus*, B=MQ2 against *A. niger*, C=MQ3 against *F. oxysporum*, D=MQ4 against *A. niger*, E=MQ5 against *A. niger*, F=MQ6 against *A. flavus,* G=MQ7 against *F. oxysporum*, H=MQ8 against *F. oxysporum*

4.6. Growth inhibition (%) by dual-culture assay

Table 4.5: Growth inhibition (%) by *Bacillus* strains MQ7 and MQ8 against selected fungal phytopathogens

Pathogens	Growth inhibition (%)-MQ7 (mean)	Growth inhibition (%)-MQ8 (mean)
A. flavus	28.71±0.25	25±0.58
F. oxysporum	50±0.5	46±0.5

Each value represents mean, ± indicates Standard deviation (SD)

Colony diameter (mm) of fungus inoculated with *Bacillus* strain was compared with fungal plug inoculated alone on SDA plate. 28.71% and 25% inhibition of *A. flavus* was observed in case MQ7 and MQ8 respectively. Moreover, 50% and 48% growth inhibition was observed in case of *F. oxysporum* by MQ7 and MQ8 respectively.

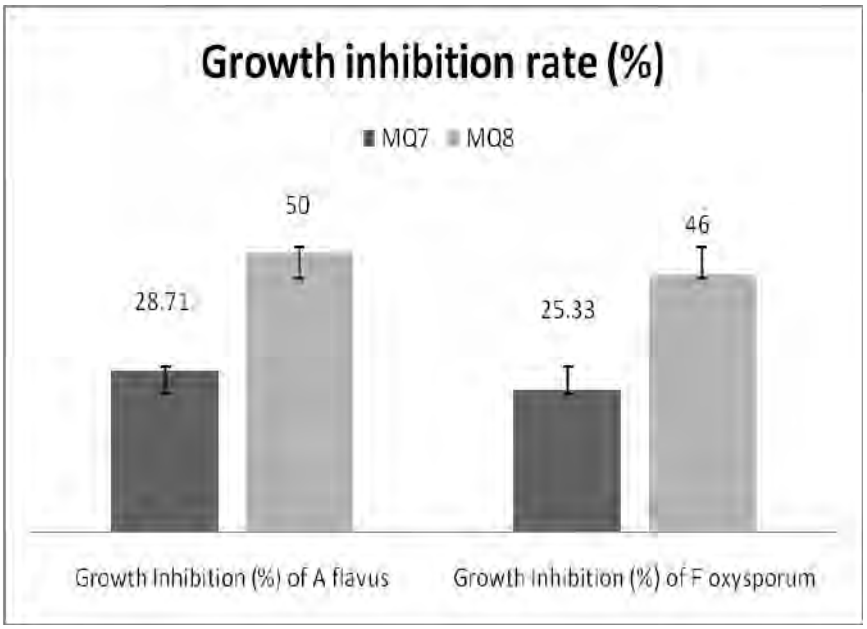


Figure 4.6: Growth inhibition(%) of fungal phytopathogens by *Bacillus* strains MQ7 and MQ8

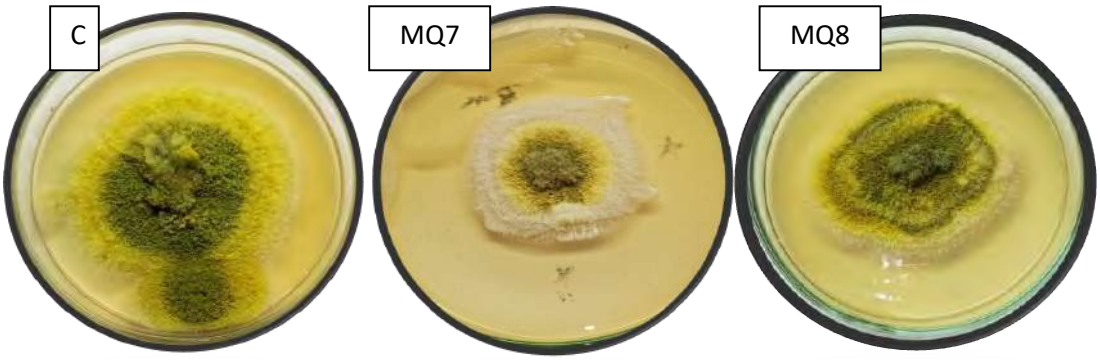


Figure 4.7: Measurement of colony diameter of *A. flavus* in presence of *Bacillus* strains MQ7 and MQ8, (C= control, fungus without *Bacillus* strains)

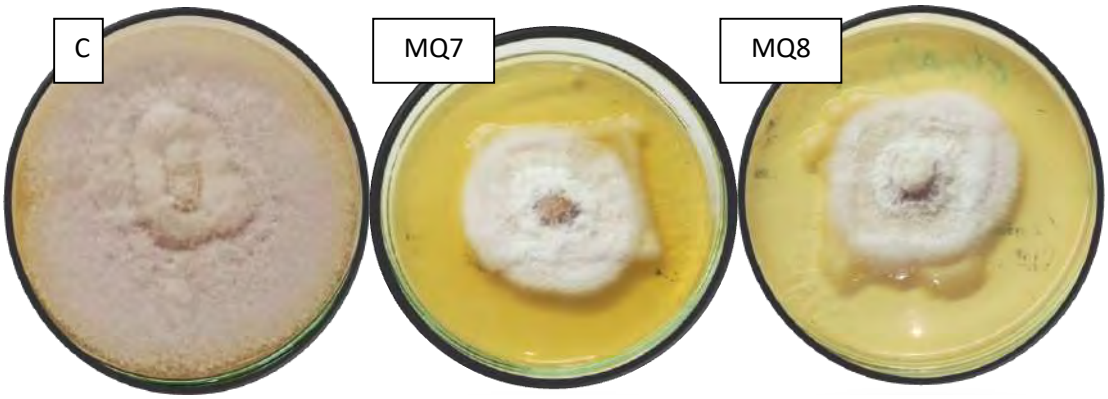


Figure 4.8: Measurement of colony diameter of *F. oxysporum* in presence and absence of *Bacillus* strains MQ7 and MQ8, (C= control, fungus without strains)

4.7. Detection of Hydrolytic enzyme produced by *Bacillus*

Selected *Bacillus* strains MQ7 and MQ8 manifested positive results for protease, amylase and endoglucanase production . Zone of hydrolysis observed in case of protease assay are 50mm by both strains. In amylase assay 10mm and 12mm zones were shown by MQ7 and MQ8 respectively. Moreover, 40mm (MQ7) and 30mm (MQ8) of zones were manifested in endoglucanase assay.

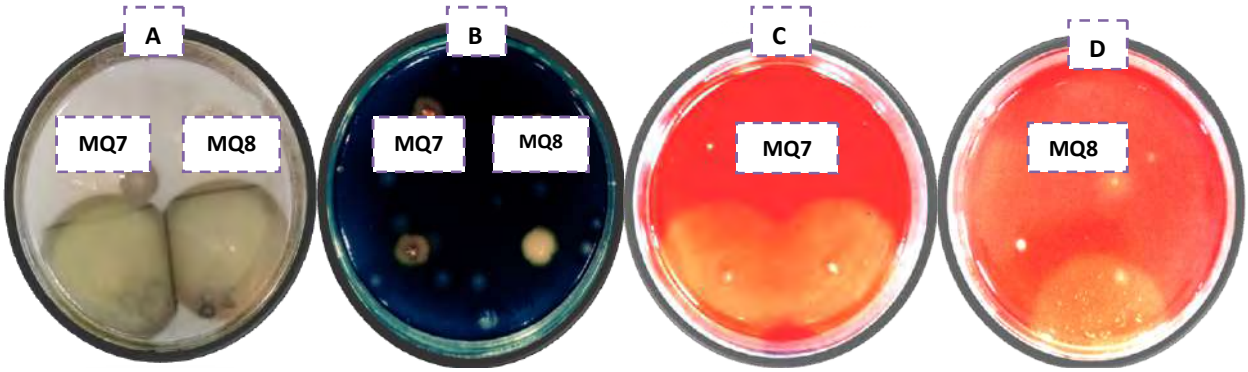


Figure 4.9: Zone of hydrolysis produced by *Bacillus* strains MQ7 and MQ8 in protease, amylase and endoglucanase enzyme assays. A= Zones indicating protease activity, B=Amylase activity and C,D = Endoglucanase activity by strains MQ7 and MQ8

4.8. Plant growth promoting traits of selected *Bacillus* strains

4.8.1. Phosphate solubilization, Indole Acetic Acid production and HCN Production

Selected *Bacillus* strains MQ7 and MQ8 manifested positive results for phosphate solubilization, Indole Acetic Acid production and HCN production. In Table 4.6 Phosphate solubilizing Index of selected strains is shown.

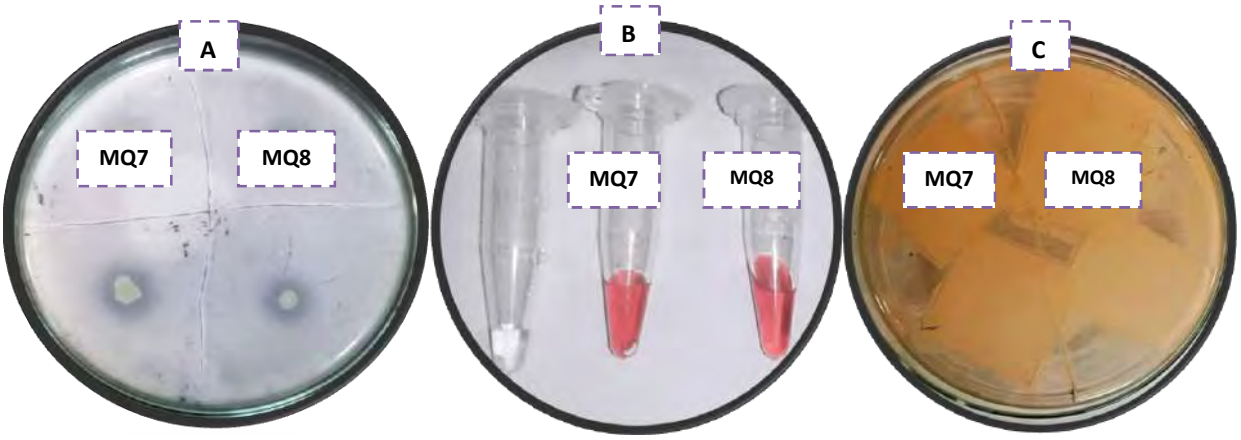


Figure 4.10: A=Zones indicating phosphate solubilization by *Bacillus* strains MQ7 and MQ8, B= Indole Acetic Acid production, C=HCN production test

Table 4.6: Phosphate solubilizing index (PSI) of *Bacillus* strains MQ7 and MQ8

Strain Code	Colony diameter-CD (cm)	Halo-zone-HZ (cm)	PSIvalue =CD+HZ/CD(cm)
MQ7	0.7±0.2	1.1±0.2	2.6
MQ8	0.5±0.1	0.9±0.2	2.8

Each value represents mean, ± indicates Standard deviation (SD)

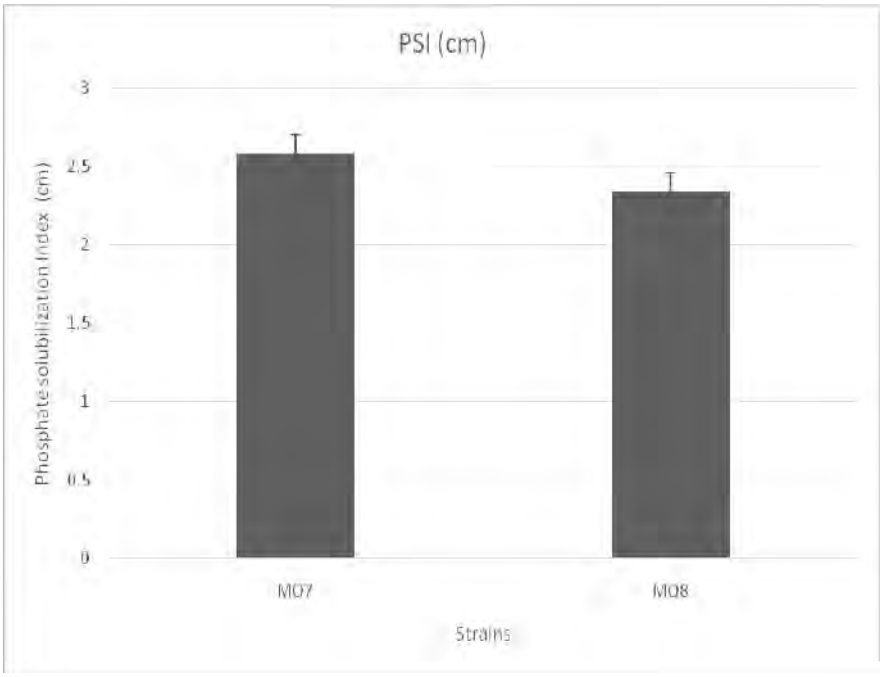


Figure 4.11: Phosphate solubilizing index (PSI) of *Bacillus* strains MQ7 and MQ8

4.8.2. Biofilm formation

Selected strains MQ7 and MQ8 showed position results for biofilm formation by showing growth on Congo red Agar. Results can be seen in figure 4.12.

Figure 4.12: Growth of *Bacillus* strains MQ7 and MQ8 on congo red agar indicating biofilm formation ability

4.9. Gel electrophoresis for DNA detection

For the confirmation of presence of genomic DNA gel electrophoresis was performed to visualize the bands. Bands appeared when seen using a UV transilluminator at high and low resolution. Results can be seen in Figure 4.13.

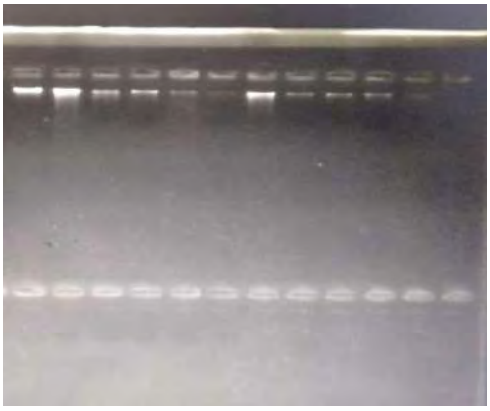


Figure 4.13: DNA bands after extraction of DNA

4.10. Polymerase Chain Reaction (PCR) of lipopeptide biosynthetic genes

PCR of strains MQ7 and MQ8 showed positive results for the presence of fengycin gene fenD by showing bands in the range of 420 to 450bp. Bands are shown in the Figure 4.14.

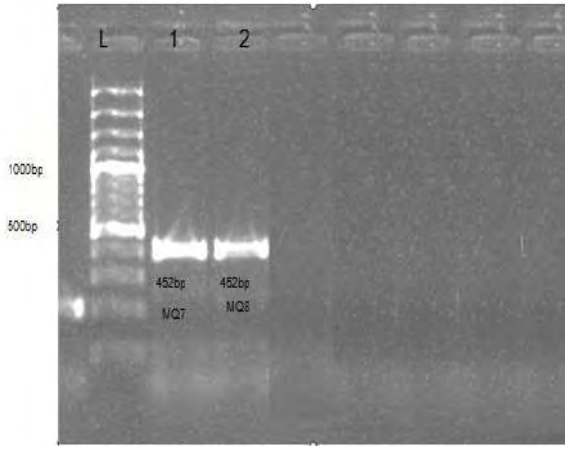


Figure 4.14: PCR based detection of lipopeptides biosynthetic genes. Bands by strains MQ7 and MQ8, indicating presence of fenD gene (fengycin lipopeptide)

4.11. Characterization of lipopeptides using FTIR

The type of lipopeptides was examined by the peaks in the graph at different functional groups using the transmittance spectra of crude extract of MQ7 strain. The standard peaks are listed in the table below (Tareq et al., 2014). The wavelengths ranged from 515 to 4000 cm-1.

Table 4.7: Reference table for functional groups in metabolite extract for FTIR

Peaks at various wavelength(cm-1)	Functional Groups
3300	Amino (N-H) stretch
2860-2930	Aliphatic side chain stretch
1735-1739	Lactone-Carbonyl absorption
1650	Peptide Bond (CO-N)
1450-1470	Methylene/methyl bond
1370	Aliphatic (C-H)bond

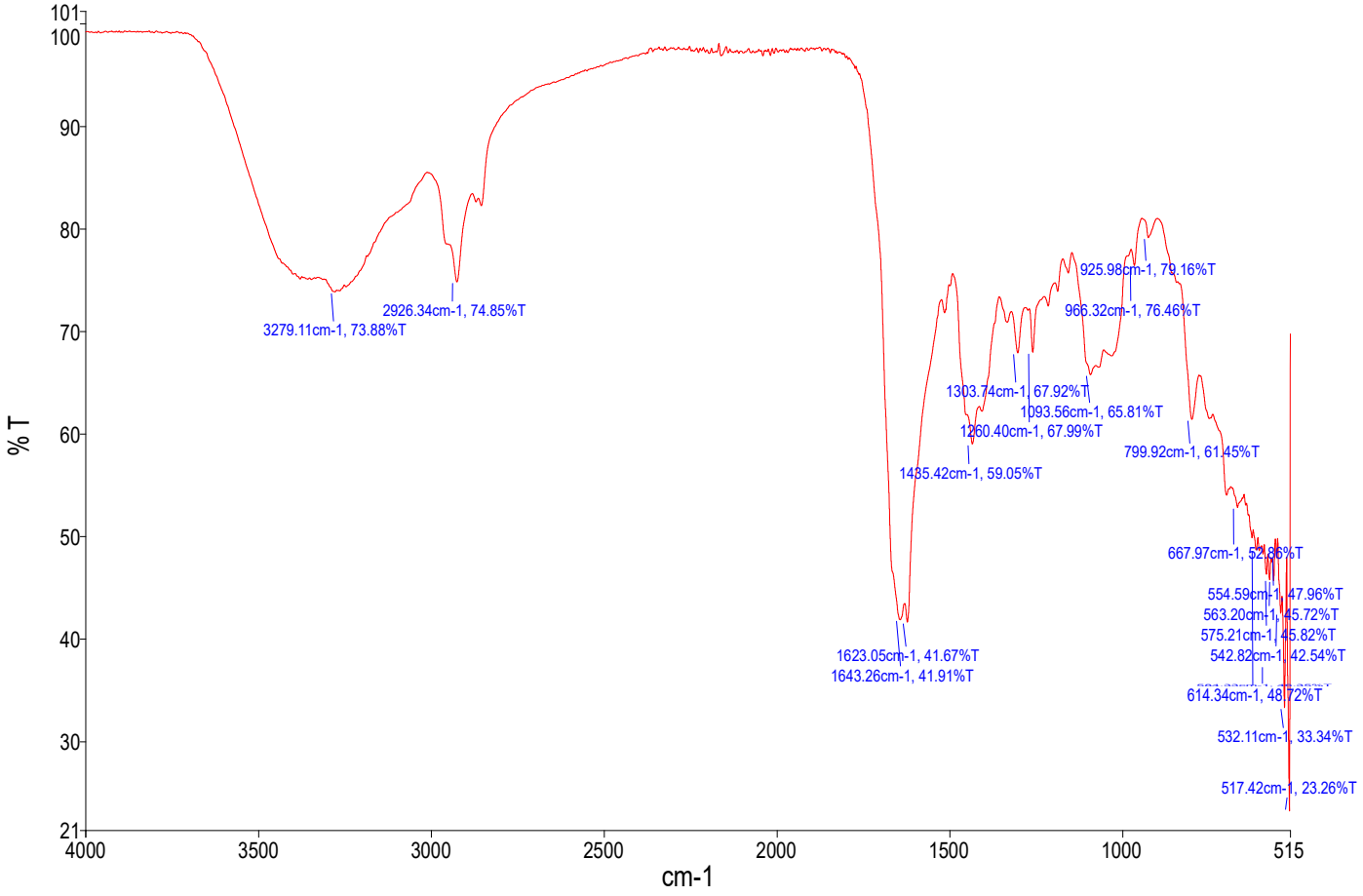


Figure 4.15(a): Transmittance spectra of crude extract of MQ7 FT-IR analysis

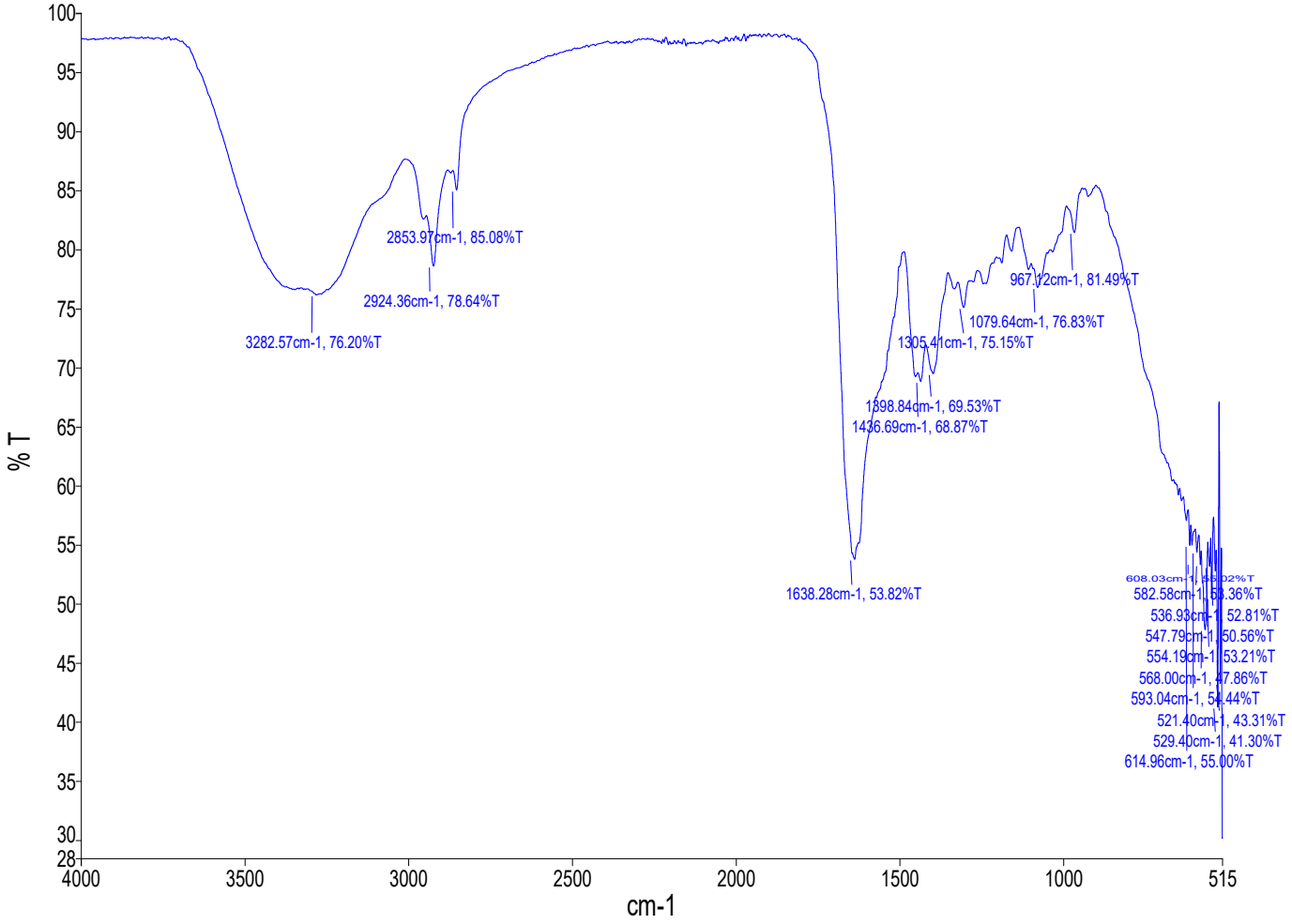


Figure 4.15(b): Transmittance spectra of crude extract of MQ8 FT-IR analysis.

4.12. Greenhouse experiment for anti-fungal (*Fusarium oxysporum*) and PGPR activity of selected *Bacillus* strains MQ 7 & MQ8

The isolated strain of *F. oxysporum* is pathogenic. Maize seeds infected with *F. oxysporum* were treated with fungicides and *Bacillus* strains. The experiment was done in completely randomized design with three replication and total 42 experimental units. In order to measure the seedling vigour index, which indicates the state of a plant's health, three guarded plants per experimental unit were chosen for data collection. Minitab was used for data analysis. In sterilized soil, disease caused by *Fusarium oxysporum* was significantly treated in the presence of the selected strains MQ7 and MQ8. Root length, shoot length, Wet mass and Dry mass were appreciably increased with chlorophyll content as well indicating plant growth promotion. Moreover, significant

disease treatment was observed in case of non-sterilized soil. Parameters including Seedling vigour index (SVI), Growth promotion (%) and Disease incidence (%), are shown in Figure 4.19, 4.23, and 4.24 respectively.

Figure 4.16: Pot experiment on *Zea maize* plant in autoclaved soil

Figure 4.17: Pot experiment on *Zea maize* plant in unautoclaved soil

Figure 4.18: Effect of different treatments *on Zea maize* plant infected with *F oxysporum*. Treatments from left to right: T1= Sterilized seed (negative control), T2= *Fusarium oxysporum(FO)*-positive control, T3= MQ7, T4=MQ8 ,T5=MQ7+FO ,T6=MQ8+FO ,T7=CFS-MQ7 ,T8=CFS-MQ8 ,T9= CFS-MQ7+FO,T10=CFS-MQ8+FO ,T11=MQ7+MQ8 ,T12= MQ7+MQ8+FO,T13= FO+commercial fungicide 1 (pyranazole) ,T14=FO+ commercial fungicide 2 (fosetyl-aluminium)

Table 4.8: Effects of selected *Bacillus* strains and commercially available fungicides in *Zea maize* plant infected with *Fusarium* specie

Treatments	Root length (cm)	Shoot length (cm)	Germination %	Seedling vigor index (SVI)	Disease incidence %	Disease control %	Percent growth increase %
Sterilized seed	12.33±0.57CD	52.33±0.58AB	100±0A	6466±115CDE			
MQ7	19.33±1.15A	58.66±1.15A	100±0A	7799±200A			
MQ8	17±1.00AB	58.67±2.30A	100±0A	7567±251AB			
Fusarium oxysporum(FO)	7±1.00E	35±3.60C	77.25±20.00B	3266.34±400F	49.48		-97.75
MQ7+ FO	17±1.00AB	55±1.00AB	100±0A	7200±0ABCD	-22.47	145.41	10.19
MQ8+FO	13.33±0.57CD	55.33±2.51AB	100±0A	6866±305ABCDE	-12.24	124.73	5.8
CFS-MQ7	14.33±0.57BC	54.66±0.58AB	100±0A	6899±0ABCDE			
CFS-MQ8	14.33±1.52BC	54±3.60AB	100±0A	6833±208ABCDE			
CFS-MQ7+ FO	12.66±0.57CD	47±6.08B	100±0A	5966±577E	15	71.11	-8.38
CFS-MQ8+FO	11.33±1.53D	48.66±1.15AB	100±0A	5999±173E	14.29	71.12	-7.78
MQ7+MQ8	17.66±0.57A	55.67±5.50AB	100±0A	7333±602ABC			
MQ7+MQ8+F0	14.33±0.57BC	50.67±6.02AB	100±0A	6500±600BCDE	_1.04	102.1	0.52
FO+ pyranazole	11.66±0.57CD	50.33±1.52AB	100±0A	6199±200DE	8.17	83.48	-4.3
FO+fosetyl-aluminium	12.66±0.57CD	50±5.00AB	100±0A	6266±550CDE	6.12	87.63	-3.1

Each value represents mean, ± represents standard deviation (SD), $p \leq 0.05$

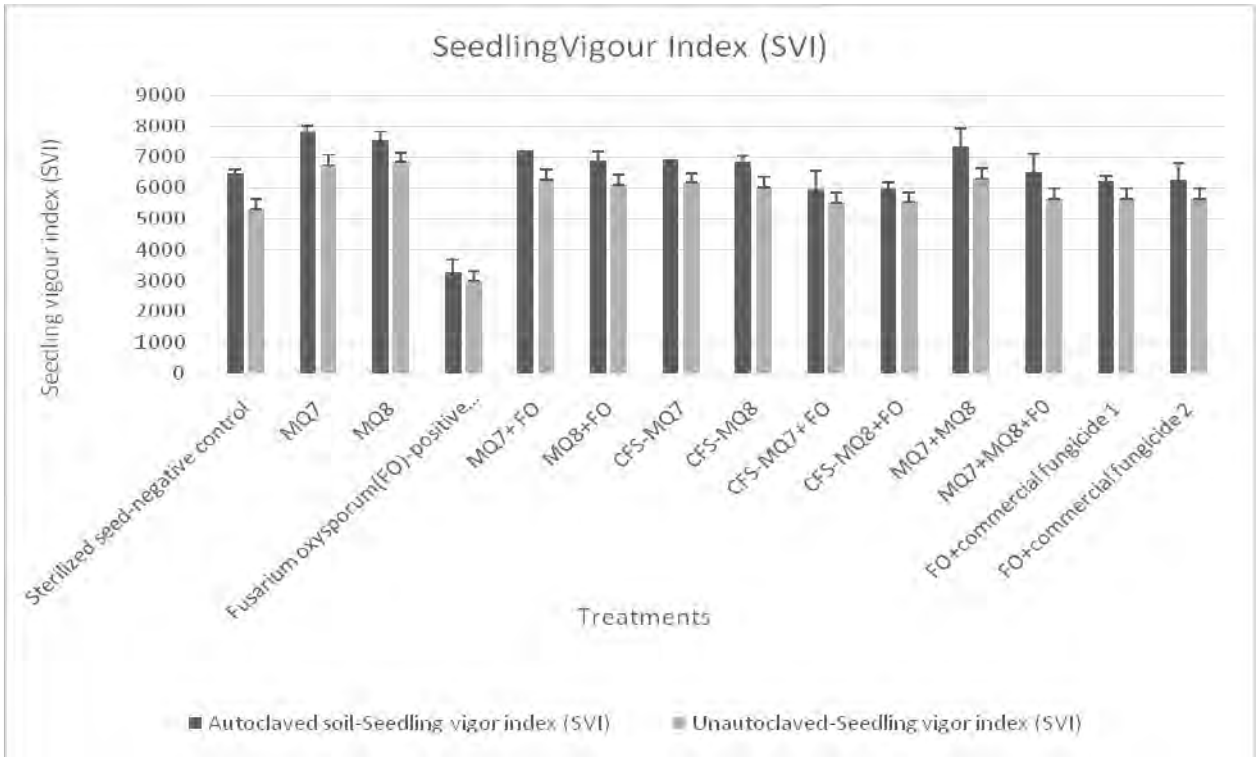


Figure 4.19: Comparison of Seedling Vigour Index (SVI) in *Zea maize* after different treatments in autoclaved and unautoclaved soil. (Error bar is indicating standard error).

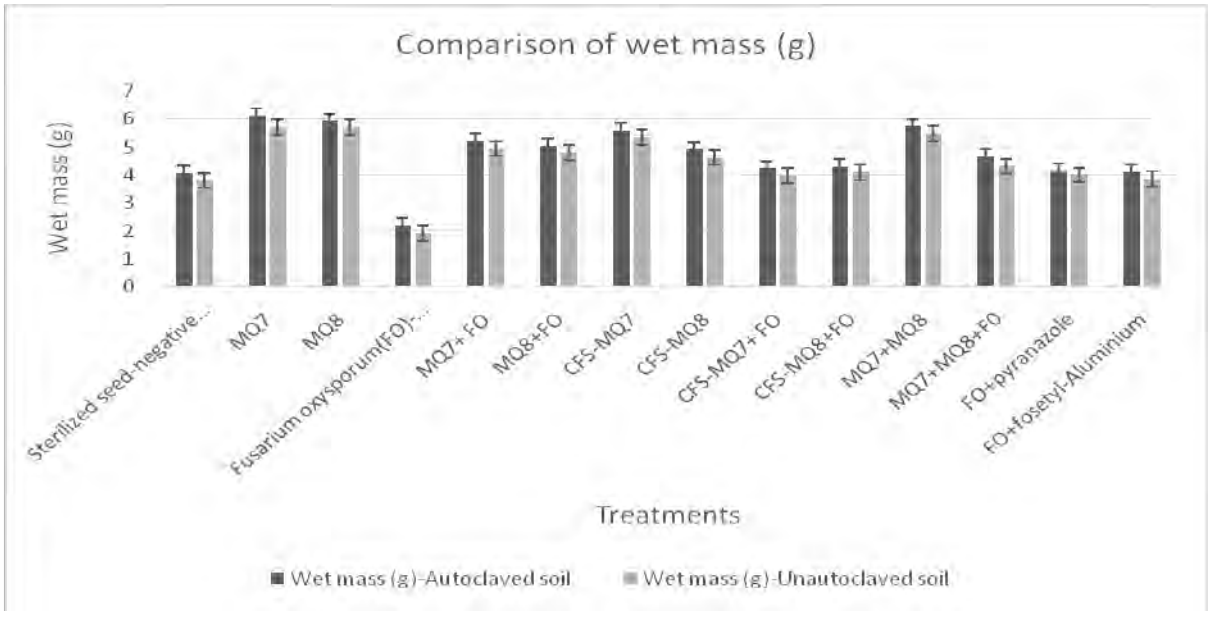


Figure 4.20: Comparison of Wet mass(g) in *Zea maize* after different treatments in autoclaved and unautoclaved soil. (Error bar is indicating standard error).

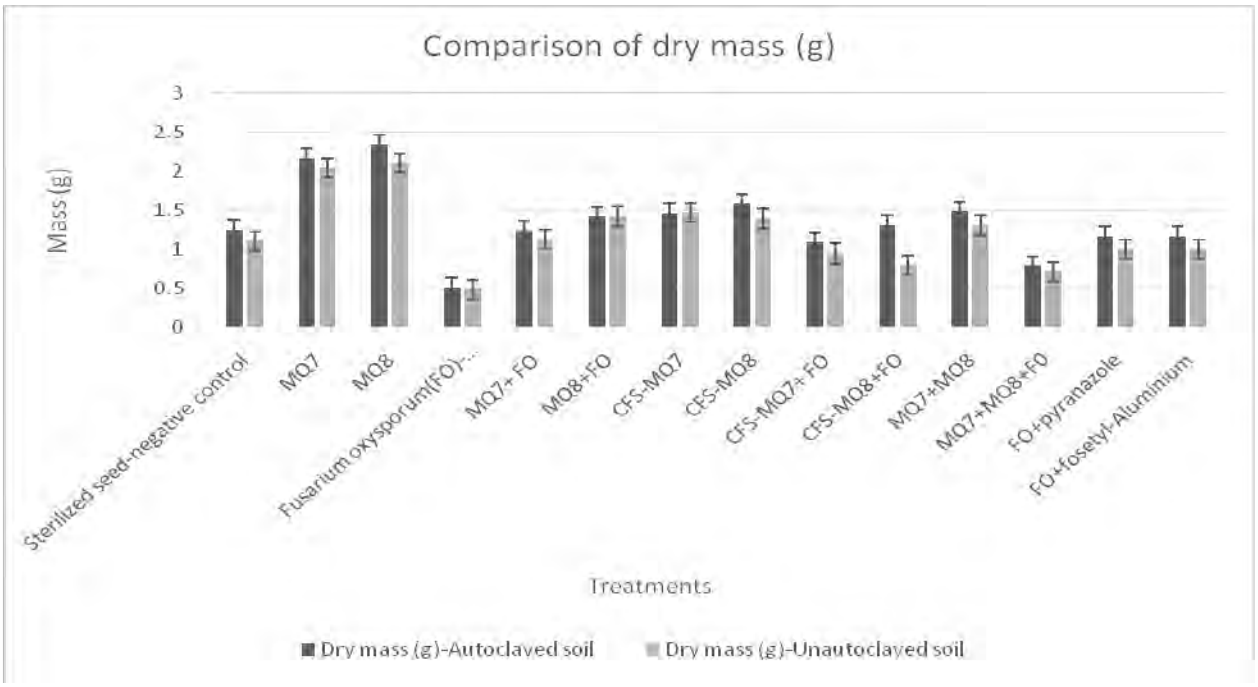


Figure 4.21: Comparison of Dry mass (g) in *Zea maize* after different treatments in autoclaved and unautoclaved soil. (Error bar is indicating standard error).

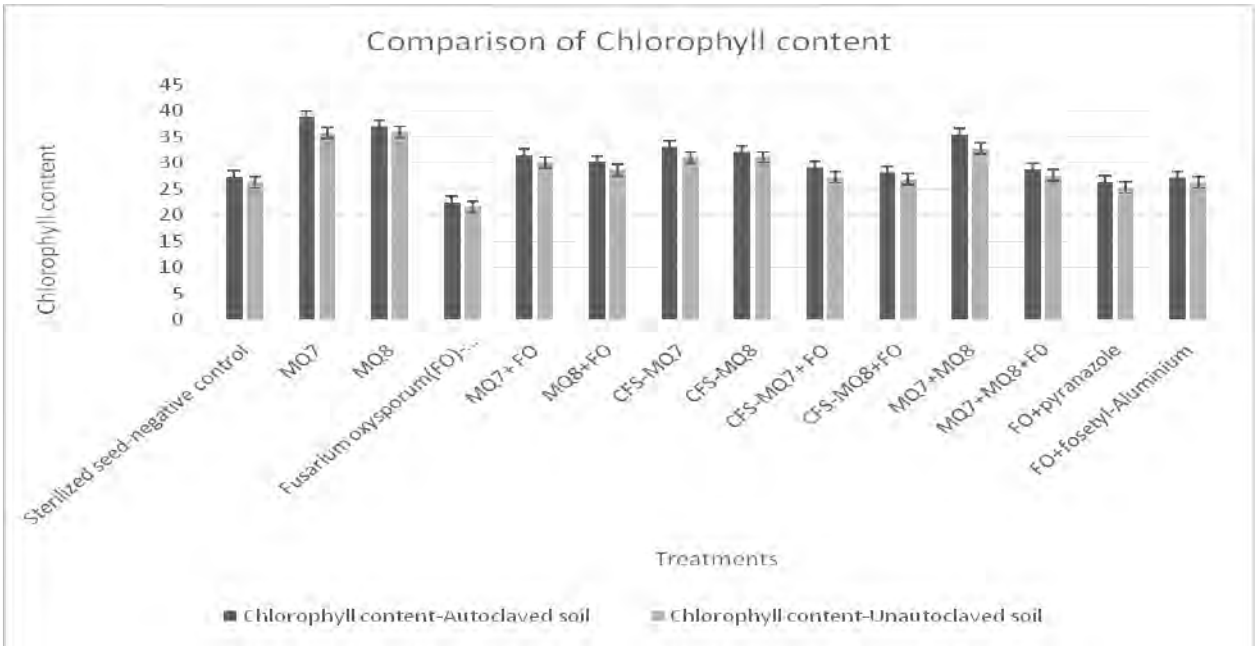


Figure 4.22: Comparison of Chlorophyll content in *Zea maize* after different treatments in autoclaved and unautoclaved soil. (Error bar is indicating standard error).

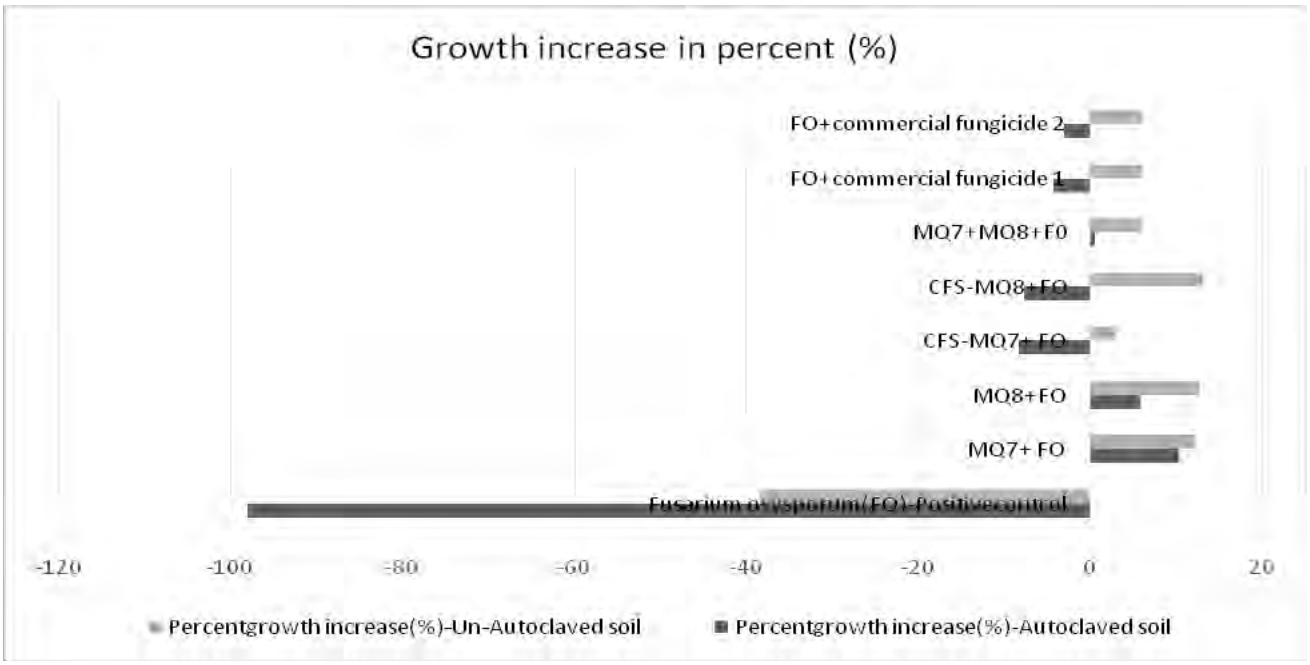


Figure 4.23: Growth increase (%) in *Zea maize* by different treatments in autoclaved and unautoclaved soil

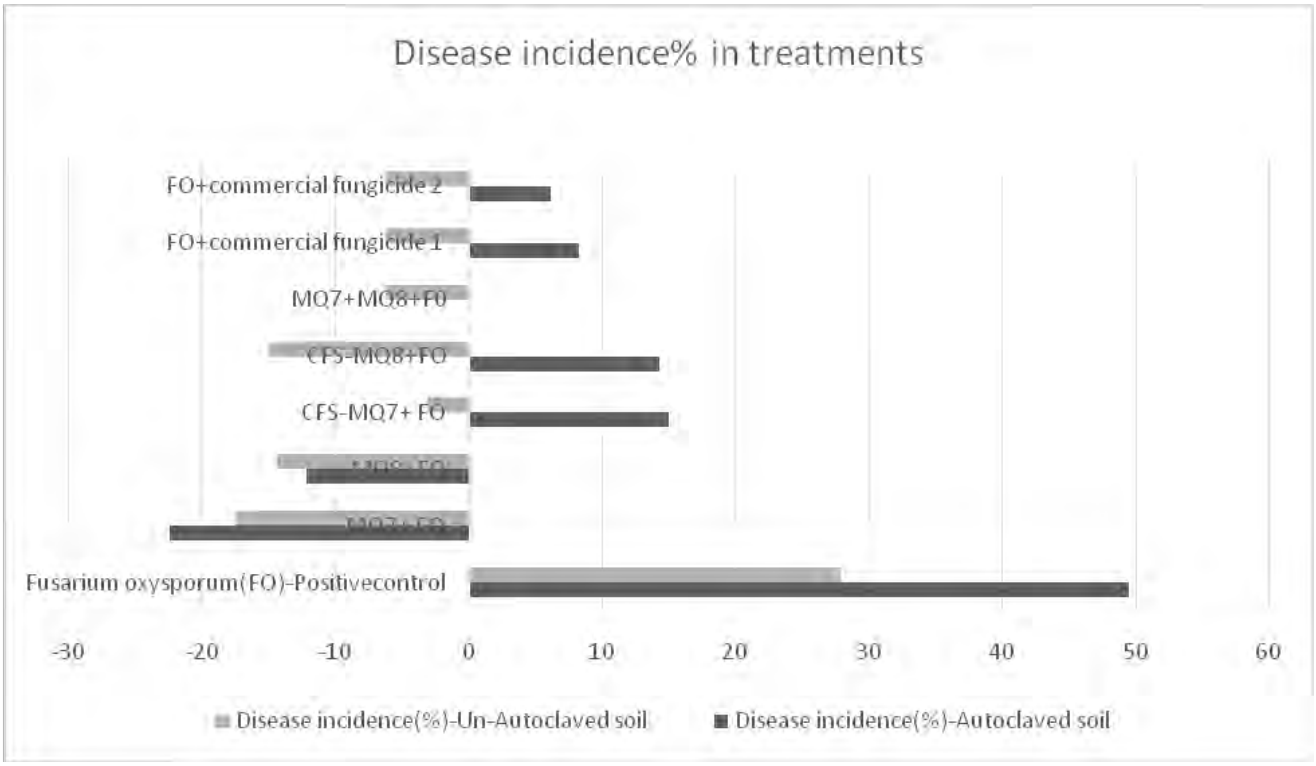


Figure 4.24: Disease incidence (%) in *Zea maize* by different treatments in autoclaved and unautoclaved soil

4.13. Pilot scale experiment to investigate effects of selected *Bacillus* strains MQ 7 & MQ8 on plant infected with *Aspergillus flavus*

The isolated strain of *A. flavus* was non-pathogenic. Maize seeds infected with *A. flavus* were treated with fungicides and *Bacillus* strains. The experiment was done in completely randomized design with three replication and total 42 experimental units. In order to measure the seedling vigour index, which indicates the state of a plant's health, three guarded plants per experimental unit were chosen for data collection. Minitab was used for data analysis. Growth was enhanced in the presence of selected *Bacillus* strains MQ7 and MQ8. Seedling vigour index (SVI) is shown in Figure 4.26

Figure 4.25: Effect of different treatments *on Zea maize* plant infected with *A flavus*. Treatments from left to right: T1= Sterilized seed (negative control), T2= *Aspergillus flavus(AF)*-positive control, T3= MQ7, T4=MQ8 ,T5=MQ7+AF ,T6=MQ8+AF ,T7=CFS-MQ7 ,T8=CFS-MQ8 ,T9= CFS-MQ7+AF,T10=CFS-MQ8+AF ,T11=MQ7+MQ8 ,T12= MQ7+MQ8+AF,T13= AF+commercial fungicide 1 (pyranazole) ,T14=AF+ commercial fungicide 2 (fosetyl-aluminium)

Table 4.9: Effects of selected *Bacillus* strains and commercially available fungicides in *Zea maize* plant infected with *Aspergillus* specie

Treatments	Root length(g)	Shoot length(g)	Germination %	Seedling vigour index(SVI)
Sterilized seed-negative control	11.33± 0.57CD	44.33±3.00A	100.00±0A	5566.67CDE
MQ7	17.33±1.00A	54.67±2.50AB	100.00±0A	7200.00A
MQ8	17.00±0.57A	53.00±2.00AB	100.00±0A	7000.00AB
A. flavus(AF)-positive control	10.33±0.25CD	51.67±1.00AB	100.00±0A	6200.00DE
MQ7+ AF	14.00±1.00AB	53.33±2.25AB	100.00±0A	6733.33ABCD
MQ8+AF	13.00±0.57AB	52.33±2.00AB	100.00±0A	6533.33ABCDE
CFS-MQ7	14.33±0.57AB	54.33±1.00B	100.00±0A	6866.67ABCDE
CFS-MQ8	14.00±1.00AB	52.33±0.25AB	100.00±0A	6633.33ABCDE
CFS-MQ7+ AF	13.00±1.00AB	55.33±0.45AB	100.00±0A	6833.33ABCD
CFS-MQ8+AF	12.67±1.00AB	56.33±0.50AB	100.00±0A	6900.00BCDE
MQ7+MQ8	15.00± 0.57AB	56.00±2.00AB	100.00±0A	7100.00ABC
MQ7+MQ8+AF	12.00±0.57CD	53.00±1.25AB	100.00±0A	6500.00CDE
AF+commercial fungicide 1	11.00±0.25CD	40.67±0.56A	100.00±0A	5166.67E
AF+commercial fungicide 2	10.33±0.56CD	42.00±1.50A	100.00±0A	5233.33E

Each value represents mean, ± represents standard deviation (SD)

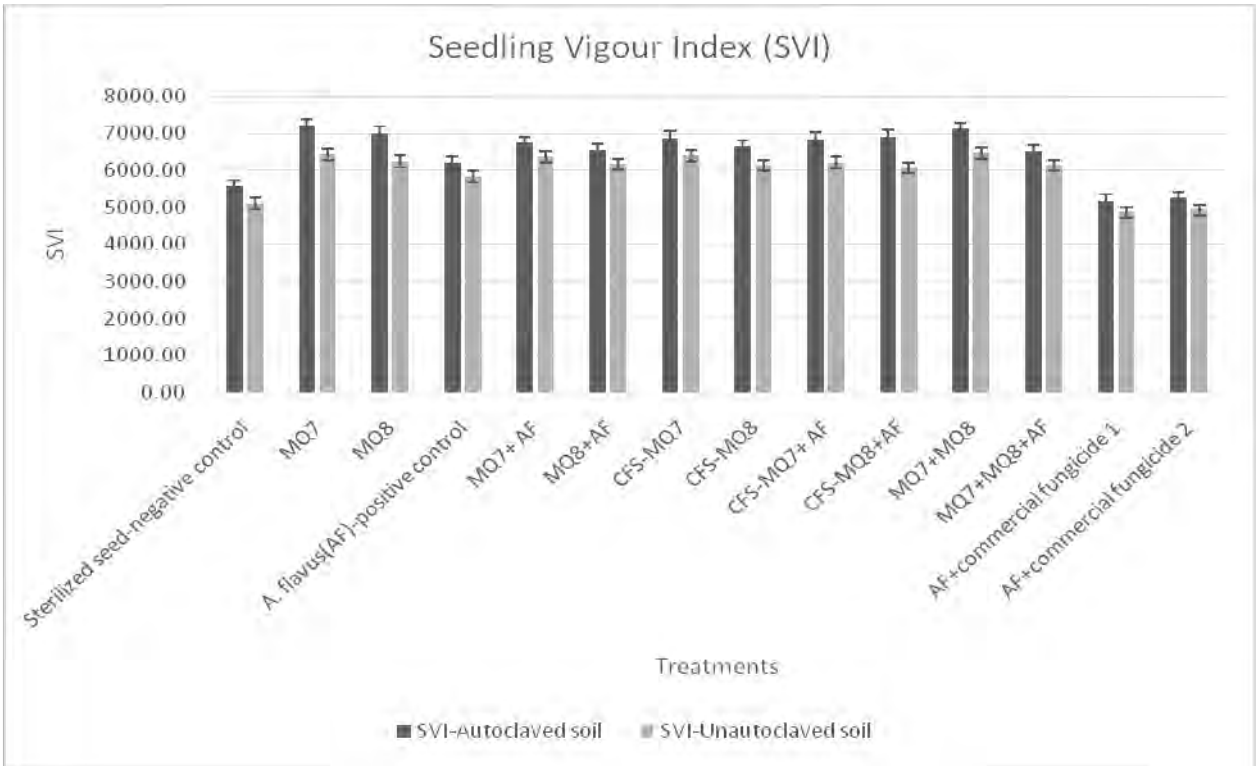


Figure 4.26: Comparison of Seedling Vigour Index (SVI) in *Zea maize* by different treatments in autoclaved and unautoclaved soil. (Error bar is indicating standard error).

5. Discussion

Since the beginning of the modern era, there has been an increase in the use of microorganisms and their metabolic products they produce to improve plant yield by reducing the negative impacts of phytopathogens. Those involved in this field are increasingly appreciating how naturally occurring microbial agents can manage plant diseases while also improving agricultural output. Due to distinct metabolic characteristics, including the ability to produce a variety of antimicrobial compounds and its ability to generate endospores, the *Bacillus* genus is frequently more efficient than other possible bioactive bacteria. *Bacillus* can reduce disease through several different processes, including parasitism, antibiosis, competition for nutrients and space, or by direct induction of systemic resistance in host plants. Therefore, there is an immense demand for active biocontrol agents that not only assist in the fight against pathogens, but also has no risks to the environment (Khan et al., 2022).

The objective of the present study was to isolate *Bacillus* strains with the potential to produce antimicrobial compounds and plant growth promoting traits. The intent was attained by isolating bacterial strains from the rhizospheric region of different agriculture fields of Charsada, KPK. 34 different strains were isolated via enrichment and serial dilution method and out of 34, 8 strains were subjected to further experimentation based on their initial antifungal screening. In the preliminary screening using the point/spot inoculation method, 8 *Bacillus* strains including MQ1, MQ2, MQ3, MQ4, MQ5, MQ6, MQ7 and MQ8 with significant antifungal activity against chosen fungal phtopathogens were selected. Out of 8, two strains; MQ7 and MQ8 were selected for detailed characterization owing to their ability to be effective against all 4 pathogens (*Aspergillus flavus, A. niger, A. fumigates and Fusarium oxysporum*). The experimentation process was done in over a year, therefore strains that showed significant antifungal activity in multiple replicates as well as in different seasons and temperature conditions (summer and winter), were further subjected to detailed characterization. MQ7 and MQ8 manifested the best antifungal performance throughout.

The findings of the current study demonstrated that bacteria with anti-fungal activity against a pathogenic *Fusarium* species cause disease in *Zea maize* plant, and also have antagonistic capabilities towards *Aspergillus flavus* which cause post-harvest disease in maize kernals.

Bacillus species showed growth inhibition of *Fusarium sp*. ranging from 46% to 50% in dual culture experiment. According to Miguel et al. (2016), antagonistic *Bacillus* species isolated from soil prevented *F. equiseti* (2.15 to 71.55%) and *F. solani* (3.76 to 69.16%) from growing their mycelium. *A. flavus's* mycelial growth was significantly (25–29%) suppressed by selected *Bacillus* species.

Plant growth promoting rhizospheric bacteria (PGPR), have been known for their biocontrol potential against phytopathogens. Especially, genus *Bacillus* has been recognized in various studies for the said purpose. PGPRs produce such antimicrobial compounds and hydrolytic enzymes including proteases, amylases and glucanases , which act upon fungal cell wall components and inhibit growth of fungal phytopathogens including; *Fusarium oxysporum, Aspergillus niger , Aspergillus flavus* and many others. This is one of the significant traits required for the biocontrol. In the current study MQ7 and MQ8 manifested zones of hydrolysis when checked for protease, amylase and endoglucanase activity, indicating their potential to release mentioned enzymes, which further confirms their antifungal role.

In preliminary qualitative analysis, MQ7 and MQ8 exhibited various plant growth promoting traits including IAA production, HCN production, phosphate solubilization, and Biofilm formation. MQ7 and MQ8 were grown in NBRIP media to evaluate their ability to solubilize insoluble tri calcium phosphate into its soluble form. Phosphate solubilization is one of the main features required for the plant growth enhancement because of its bio-unavailability in soil. MQ7 and MQ8 showed solubilizing potential with 2.6±0.2 and 2.8±0.2 PSI respectively. PSI indicated by selected strains is comparable with the study done by javorekova et Al., 2021, with approximately 3cm of zone reported. Phosphate solubilizing bacteria (PSBs) have the potential to release phosphatases enzymes and some organic acid to convert insoluble form of phosphates into soluble form of phosphorus which is then bioavailable and can be utilized by the plants, consequently leading to the better plant growth.

Selected strains MQ7 and MQ8 shown to have Indole Acetic Acid (IAA) producing capability, which is plant growth hormone, commonly known as auxin. IAA has its role in cell division, cell enlargement, root development and growth leading to the better nutrient absorption, which is consequently beneficial for the enhanced growth of plant.

To test the potential of different *Bacillus* strains to form biofilm, Congo red agar was used. The colonies of every species were found to be dry and reddish. According to Romero et al. (2010), TasA is an amyloid protein that forms amyloid fibers that serve to create biofilm and provide strength to the extracellular matrix. Congo red dye is bound by TasA, giving the colony a red appearance. Mutant *Bacillus* species without the TasA gene are unable to generate biofilm and their colonies don't seem red. All of the *Bacillus* species' colonies were seen to be red, which suggests that Cellulose Congo Red Agar (CCRA), in addition to cellulose hydrolysis, can also be used in place of Congo red agar with brain heart infusion to test the capacity of wild-type *Bacillus* species to produce biofilm. By assisting the *Bacillus* in acquiring nutrients and space as well as improving nutrient mobility within the rhizosphere, the extracellular matrix serves to keep plant diseases from colonizing. *Bacillus* subtilis (3610) is less successful at controlling the tomato disease *Rhizoctonia solanacearum* due to restricted matrix synthesis, according to Chen et al. (2013). In the current study *Bacillus* isolates were shown to be biofilm producers, hampering the growth of test fungus in vitro and indicating that they may effectively colonise the surfaces of plants to reduce disease.

The main methods used by PGPR to prevent pathogens from invading host plant tissues are the synthesis of antibiotics and antifungal compounds. Two non-ribosomal peptide (NRP) producing genes were screened using PCR analysis in order to examine the antibiosis mechanisms of the MQ7 and MQ8 strains. Iturin C and fengycin D genes can both be amplified using the primers fenD-F/fenD-R and ituC-F/ituC-R. Fengycin and iturin effectively inhibit the growth of a variety of plant diseases due to their strong antifungal capabilities. The selected *Bacillus* strain MQ7 and MQ8 manifested positive results for the lipopeptide (fengycin D) gene. According to study done by Tapi et al.'s (2010), the estimated gene size was amplified

The FTIR analysis of the crude extract revealed cyclic lipopeptide peaks, including iturin, fengycin, and surfactin. The metabolite architecture's inclusion of aliphatic side chain of was confirmed by the infrared spectra of MQ7 crude lipopeptide content, which showed strong bands at 3264.85cm-1 and 2934.29 cm-1 indicating Amino (N-H) stretch and Aliphatic side chain stretch respectively. Peaks at 1709.42 cm-1 suggests lactone – carbonyl absorption and 1649.70 cm-1 indicates peptide bond, and strong bands at 1535.24 cm-1 and 1389.07cm-1 respectively proved the presence of Amine primary group (N-H) and (C–O) bonds. These bands exhibit a

lipopeptide-like chemical structure, including hydrophobic aliphatic chains and hydrophilic peptide moieties. Comparing with previously published research reveals the existence of iturin (Narendrakumar et al., 2016), fengycin (Wei and Yang, 2010), and surfactin (Veshareh and Nick, 2018; Sivapathasekaran et al., 2010). It can be predicted that the molecule may naturally be a lipopeptide based on the findings of current investigation and comparison with the published spectra of lipopeptide in literature (Wu et al., 2019).

B.subtilis antifungal abilities can be determined by in vitro experiments, yet in vivo testing of its biocontrol effectiveness is equally vital. The MQ7 and MQ8 strains are exceedingly effective at preventing *Fusarium* spp.-caused root rot, according to in vivo tests done on *Zea maize* crops. In current study isolated *Bacillus* strains MQ7 and MQ8, along with their metabolites (cell-free supernatant), substantially decreased the incidence of disease in autoclaved soil and increased plant growth by 10.13 and 5.8%, in comparison to controls, respectively. Seed germination, however, did not differ significantly. Moreover, it was also demostrated by Cavaglieri et al.. (2005) that, *Bacillus* species greatly suppress *Fusarium* disease in *Zea maize* plants and promote plant growth in sterilized soil. According to Posada et al. (2016), compared to the control, Musa plants using cell-free supernatants that were either obtained from vegetative cells or endospore had significantly increased plant length and dry weight. The increase in plant growth in case of seeds treated with *Bacillus* when compared to metabolites suggests that either plant growth-promoting compounds may be produced or that the plants may be assisted in acquiring nutrients by forming a connection through the development of a biofilm during seedling establishment. Additionally, CFSs has some limitations as soil fertilizers by metabolite liability (sensitive to strong bases, acids and temperature) (Pellegrini et al., 2020). The significantly decreased biomass and chlorophyll concentrations in the seedlings suggest that the decreased plant growth in soil infested with *Fusarium* may be caused by induction of stress in the seedlings.

Current research supports the findings of Baghbani et al. (2019), that states lower chlorophyll levels in *maize* when ifected with *F. verticilloides*. Our *Bacillus* strains and their CFSs considerably decreased disease incidence in non-autoclaved soil and enhanced plant growth, although SVI measure of plant development, did not improve in comparison to the negative control. According to Martinez-Viveros et al. (2010), a critical level of population density of beneficial bacteria is required to bring a major shift in plant growth. However, in soil which is

un-sterilized, rapid descent in bacterial population in comparison to autoclaved soil due to competition with native microbiota and attack of predators (protozoa and nematodes). In sterilized soil, Marschner and Rumberger (2004) also saw a further, swift recolonization of bacterial communities. These investigations support the reported variations in plant growth between sterilized and non-sterilized soils. In case of *Aspergillus flavus* no pre-harvest disease incidence was observed, however there was increase in plant growth in case of positive control *(Aspergillus flavus)* in comparison to the negative control. *Bacillus* strains MQ7 and MQ8 also enhanced the growth of plant significantly. Recent study done by Omomowo et al, (2020) also reported non-pathogenic *Aspergillus flavus* that enhance plant growth by solubilizing phosphate.

The findings of this study can be succinctly summed up as follows: *Bacillus* genus is among the most intriguing and potential candidate for use as bioactive agent, displaying broad inhibition spectrum against plant diseases. To formulate commercial biocontrol products, it is also advisable to take bio-controls into account as a green substitute for conventional pesticides. Thus, the unique idea of bio-controls will eventually find application beyond the lab, and its benefits will be visible in both the current and future crop production systems.

Conclusion

To sum up the current study, two *Bacillus* strains (MQ7 and MQ8) were isolated from soil and screened against *Fusarium* specie and *Aspergillus* species. *Fusarium sp*. is the etiological agent of *maize* root rot disease, while *Aspergillus flavus* is associated with ear rot disease with high incidence. The results indicated that the strain MQ7 exhibited excellent anti-fungal activity against *Aspergillus flavus*, *Aspergillus niger and Fusarium oxysporum* with inhibitory zones of 55mm, 56mm and 23mm respectively. In the case of strain MQ8, 35mm, 48mm, and 35mm of the zones were recorded for the aforementioned fungal isolates. The antifungal activity of the *Bacillus* strain MQ7 was comparatively higher than the strain MQ8 i.e. 50%±0.5 for *F. oxysporum*, and for *A. flavus* 28.71%±0.25. In-vivo experiments on *Zea maize* showed increase in plant growth up to 10.13% and 5.8% in the presence of strain MQ7 and MQ8 respectively, in comparison to the positive control. The selected *Bacillus* strains inhibited the mycelial growth of both pathogenic fungi. Both of the selected strains showed plant growth promoting traits including phosphate solubilization, IAA production, Biofilm formation and HCN production. Therefore, it can be concluded that the selected strains have the two in one capability (Fighting pathogen and enhancing growth) and can act as a potential alternate to the chemical pesticides and fertilizers.

Future Prospects

Plant seedling development and growth is directly influenced by soil-borne fungal pathogens. Numerous fungi can infect the seeds during germination, reducing total output and posing economic losses. By suggesting several disease prevention and plant growth enhancement techniques, *Bacillus* species can enhance seedling and plant health.

- To improve the strains, multiple mechanisms of action can be combined in single *Bacillus* specie using advanced biotechnological technologies.
- Characterization of antimicrobial substances using in-situ molecular methods is required.
- More research is needed to evaluate the efficacy of using *Bacillus* strains in combination with other biocontrol agents.
- Other mechanisms of disease prevention and plant growth enhancement can be further studied and quantified.
- Further research can be done to determine the effectiveness of different *Bacillus* strains in preventing soil- and seed-borne illnesses in other crops.
- Other parameters including; climate change, soil conditions, temperature variations, stability of biosurfactants can also be considered during field experiments to make biocontrol more holistic approach to attain sustainability.

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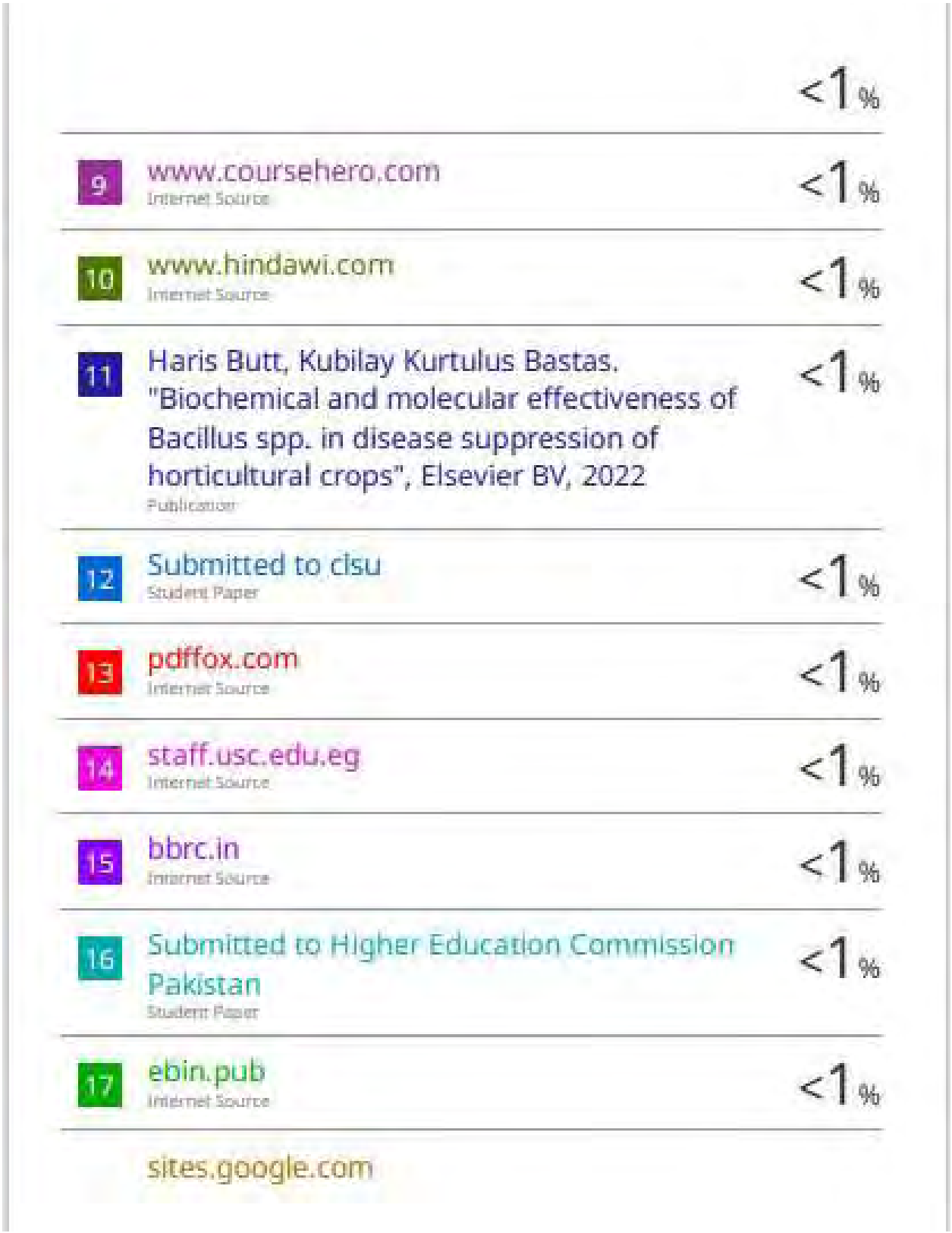

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