Isolation and Characterisation of Potential Biocontrol Agents against foliar disease of *Solanum lycosperscium*

A Dissertation for

Course code and Course Title: GBT-651 Dissertation

Credits: 16

Submitted in partial fulfilment of Masters of Science Degree

In Biotechnology

By

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Under the Supervision of

Ms. SNIGDHA SHARAD MAYENKAR

School of Biological Sciences and Biotechnology

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DECLARATION BY STUDENT

I hereby declare that the data presented in this Dissertation report entitled, "**Isolation and Characterisation of Potential Biocontrol Agents against foliar disease of *Solanum lycosperscium***" is based on the results of investigations carried out by me in the Biotechnology discipline at the School of Biological Sciences and Biotechnology, Goa University under the supervision of Ms. Snigdha Sharad Mayenkar and the same has not been submitted elsewhere for the award of a degree or diploma by me. Further, I understand that Goa University or its authorities will be not be responsible for the correctness of observations / experimental or other findings given the dissertation. I hereby authorize the University authorities to upload this dissertation on the dissertation repository or anywhere else as the UGC regulations demand and make it available to any one as needed.

A Pednekar

Ambika Sainath Pednekar

Seat no: 22P0470002

Date: 08 April 2024

Place: Goa University

COMPLETION CERTIFICATE

This is to certify that the dissertation report "**Isolation and Characterisation of Potential Biocontrol Agents against foliar disease of *Solanum lycosperscium***" is a bonafide work carried out by **Ms. Ambika Sainath Pednekar** under my supervision in partial fulfilment of the requirements for the award of the degree of **Master of Science** in the Discipline of Biotechnology at the School of Biological Sciences and Biotechnology, Goa University.

Ms. Snigdha Mayenkar
Assistant Professor
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Date: 08 April 2024

Prof. Bernard F. Rodrigues
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PREFACE

The increasing global population has resulted in a rising need for higher production of crucial vegetable crops such as tomatoes (*Solanum lycosperscium*). Tomatoes rank as the second most significant vegetable known for its versatility in consumption. It thrives in cool and warm climates. These climatic conditions, however, create an ideal environment for the development of stem, soil-borne and foliar diseases. When crops are affected by such diseases, farmers not only lose the value of their current harvest but also incur additional costs for disease management, such as fungicide application etc. leading to substantial economic losses and reduced productivity in these vital commercial crops. Although existing chemical pesticides and insecticides have effectively mitigated the severity of such diseases, they have also demonstrated adverse effects on soil, plant and human health.

Hence to overcome such undesirable consequences biocontrol agents provide a sustainable solution. Biocontrol agents offer year-round safeguarding from pathogens without toxicity to plants, are environmentally friendly, simple to produce, cultivate and control. Furthermore they enhance crop output and can be paired with bio-fertilizers hence making them a better alternative to chemical pesticides and fungicides.

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LIST OF ABBREVIATIONS

Sr. No.	Abbreviations	Full form
1	PDA	Potato Dextrose Agar
2	Mm	Milimeter
3	mL	Mililitre
4	L	Litre
5	HCN	Hydrogen Cyanide
6	Gms	Grams
7	µL	Microlitre
8	ºC	Degree Celsius
9	%	Percentage
10	Fig.	Figure
11	MBCA	Microbial Biological Agent
12	WP	Wettable powder
13	PGPR	Plant growth promoting rhizobacteria
14	AM	Arbuscular mycorhiza
15	SA	Salicyclic acid
16	JA	Jasmonic acid
17	NPK	Sodium Phosphorus Potassium
18	PIRG	Percentage inhibition of radial growth
19	CCA	Colloidal chitin agar
20	NA	Nutrient agar
21	CTAB	Cetyltrimethyl ammonium bromide
22	CAS	Crome azurol sulfonate
23	W/W	Weight by weight
24	Cm	centimetre

ABSTRACT

The tomato, a significant vegetable crop that holds versatility in its consumption. It can be eaten raw or incorporated into various dishes like soups and sauces. However, its productivity is dwindling due to a multitude of factors, both biotic and abiotic. Among these, fungal diseases such as myrothecium leaf spot exert substantial influence. While chemical pesticides are commonly employed to combat this issue, their application possess risks to human health, animal well-being, and the environment. Biocontrol agents emerge as a superior alternative, providing year-round protection against pathogens without the harmful effects associated with chemical counterparts. These agents, being environmentally sustainable and easy to produce, cultivate and manage, not only mitigate disease but also augment overall crop yield.

In this research, potential biocontrol agents sourced from the rhizosphere of healthy tomato plants were isolated and evaluated against the phytopathogen *Myrothecium* sp. The isolates exhibited inhibition of *Myrothecium* sp. growth in dual culture inhibition tests. Additionally, they demonstrated the capacity to produce cell wall-degrading lytic enzymes and bioactive compounds. Notably, among the isolates, RSSM13 showcased the highest mycelial radial inhibition (by 50.68%) in the dual culture test. Furthermore, in pot trials on 25 day-old tomato saplings, the newly isolated biocontrol agent RSSM13 exhibited efficacy comparable to that of the chemical pesticide Bendaco (carbendazim-12%w/w+mancozeb-63%ww) in disease prevention.

CHAPTER 1: INTRODUCTION

1.1 Background

Agriculture is one of the most important sectors in India that controls the economy of the country. It provides support directly or indirectly to almost 70% of Indian population and their livelihood (Jain & Goria, 2001). Agriculture, along with its various sectors, indisputably stands as the primary source of livelihood in India, particularly in its expansive rural regions. Additionally, it makes a noteworthy impact to our country's Gross Domestic Product (GDP). The significance of vegetable production is on the rise, driven by the rapid urbanization process that is generating a heightened need for crops like tomatoes and cabbage (Nagaraju et al., 2002).

Tomato (*Solanum lycospersicum*) is one of the major economical crop grown all over the world. It is known to be a significant food crop as well as cash crop for many low income farmers in tropical countries (Lal et al., 2015). It is a very versatile crop known to have products which can be eaten either raw or cooked. Tomatoes are used in making wide variety of dishes including salads, curries, ketchups, soups etc. (Borguini & Ferraz Da Silva Torres, 2009). It is has minerals such as phosphorus, calcium, iron and also vitamins like A, E. Furthermore, it serves as an outstanding reservoir of antioxidant elements such as carotenoids (especially lycopene), ascorbic acid, and phenolic compounds (Bhowmik et al., 2012). Tomato is the second most important vegetable crop and for it to flourish it requires cool and warm climate. Such cool and warm climatic conditions become ideal for spread of stem, soil borne and foliar diseases (Kumar et al., 2018).

Every year, a substantial quantity of crops is lost during both pre and post-harvest phases, primarily due to pathogen infestation. This encompasses a diverse array of pathogens, including viroids, viruses, prokaryotic bacteria, oomycetes, nematodes, and fungi. These

pathogens exhibit strong persistence in their attacks, resulting in direct and indirect losses amounting to approximately $40 billion worldwide (Lal et al., 2015; Pandit et al., 2022).

Fungal diseases are a major limiting factor for tomato production as they cause severe economic loses. Some of the fungal diseases include septoria leaf spot, seedling damping off, early blight, late blight, powdery mildew, anthracnose, southern leaf blight, verticillium wilt, fusarium wilt, buckeye rot and myrothecium leaf spot (Kumar et al., 2018; Raghavendra et al., 2023; YanJie et al., 2009).

To overcome losses caused by such diseases and thus increase the overall productivity fungicides, chemical pesticides, herbicides and insecticides have been employed. Such synthetic chemicals are known to provide defence from fungi, insects, nematodes, weeds and mites but has negative impact on the soil features and interrupt the beneficial insects from completing its life cycle. It also causes serious damages to human health depending on the route, duration of exposure and individuals health status (Koul et al., 2022; Nicolopoulou-Stamati et al., 2016). Farmworkers are exposed to these harmful chemicals either directly or indirectly, while the general population encounters them through skin contact, often due to pesticide drift during mixing. This poses significant risks to human health, including the development of conditions such as reproductive disorders, diabetes, respiratory issues neurological dysfunction and cancer (Rani et al., 2021).

Hence to overcome such adverse negative effects biocontrol agents were introduced. Use of biological control have been active from around 100 years. Biological controls refer to ordinary or genetically modified organisms such as yeasts, fungi, bacteria and endophytes, or innovative gene products utilized to manage and combat plant pathogens and related diseases. They offer cost-effective solutions, ensure year-round protection, pose no toxicity risks to plants, are environmentally friendly, straightforward to cultivate, reproduce, and

manage. Moreover, they enhance crop yields and can be combined with bio-fertilizers for synergistic effects (Koul et al., 2022).

Biological control, in essence, entails utilizing living organisms to combat specific plant diseases or pests through mechanisms such as antibiosis, competition or parasitism, for space or resources. For a pathogen or pest to flourish on a plant, it must meet three key criteria: the attacker itself (pathogen or pest), the environment, and the plant's characteristics. To encompass these principles fully, a wide-ranging definition of biological control is required, one that encompasses all levels to unlock its complete potential in disease and pest management. Thus, this expanded definition should involve the utilization of species and their by-products to control diseases and pest in crops, either by inducing hostile reactions or by fostering immunity against them (Pandit et al., 2022).

Microbial biological agents (MBCAs) are selected advantageous microorganisms that exhibit high efficacy against pathogens and can be cultivated in synthetic environments are utilized in agriculture. These carefully selected microbial biological agents are deployed in significant quantities during the growing season (Eilenberg et al., 2001). Farmers employ Microbial Biological Control Agents (MBCAs), which consist of living microorganisms registered as crop defence products manufactured by biological control companies. In certain instances, the final product may also incorporate antimicrobial metabolites generated by specific microorganisms. Some products may even exclusively contain antimicrobial metabolites without the presence of live antagonist cells (Glare et al., 2012). MBCAs can be as spray for foliar diseases or mixed with rhizospheric soil. Biocontrol agents can also be combined with chemical products with use of physical methods to increase effectiveness (Spadaro & Gullino, 2005).

Plant disease are developed due to interaction of three different elements of the disease triangle namely the pathogen, plant and environment. Biological control agents are organisms that engage with the various elements of the disease triangle in an attempt to regulate the disease. Hence understanding the working mechanism of biocontrol agents in mitigating the disease becomes important. Biocontrol agents have a baffling array of biocontrol mechanisms to control the spread of disease (Chet, 1987). The different biocontrol mechanisms involved are hyperparasitism, competition, antibiosis, secretion of lytic enzymes, plant growth promotion, and induced systemic host resistance.

Hyperparasitism- Hyperparasitism represents the most prominent and direct form of antagonism. It entails the targeted growth of a biocontrol agent toward the host organism, where it coils, attacks, and disintegrates the cell wall or membrane of the target pathogens through enzymatic activity (Junaid et al., 2013).

Competition- Competition, from a microbial standpoint, arises due to the nutrient-limited nature of plant surfaces. To colonize the phytosphere successfully, microbes must consistently vie for available nutrients. Both pathogens and biocontrol agents engage in competition for nutrients and space to establish themselves in the environment. This competitive process, known as indirect antagonism, results in the exclusion of pathogens through the depletion of their food sources and physical occupation of sites (Lorito et al., 1994). Moreover, competition can extend to essential micronutrients like iron and manganese, particularly in highly oxidized and aerated soils. Competition for micronutrients arises due to the superior nutrient uptake system of biocontrol agents, attributed to their production of iron-binding ligands known as siderophores (Nelson, 1991). This results in the sequestration of iron, rendering it unavailable to pathogens with lower siderophore production and weaker iron-binding capacity. Consequently, pathogen spread is reduced, leading to improved disease control (Junaid et al., 2013).

Antibiosis- Antibiosis atributes to the creation of low molecular weight compounds or antibiotics by microorganisms that directly inhibit the plant pathogen growth. An effective biocontrol agent is one that generates adequate masses of antibiotics in the vicinity of the plant pathogen (Gour & Purohit, 2004).

Secretion of lytic enzymes-The secretion of lytic enzymes is another mechanism employed by diverse microorganisms to restrict plant pathogen growth and activities. Many microorganisms produce and release various lytic enzymes capable of hydrolyzing a varied variety of polymers such as chitin, proteins, cellulose, and hemicellulose (Ordentlich et al., 1998).

Additionally, microbial products like hydrogen cyanide (HCN) can suppress pathogens by effectively blocking the cytochrome oxidase pathway, exhibiting high toxicity to aerobic microorganisms even at picomolar concentrations (Howell et al., 1980).

Plant growth promotion- Plant growth promotion involves biocontrol agents decreasing crop disease incidence and enhancing growth, particularly in the early stages of the life cycle, often by enabling disease escape. An excellent illustration of this is the resistance observed in solanaceous crops against damping off as they mature. Studies have shown that both fungal and bacterial biocontrol agents aid in disease management by fostering plant growth. This is achieved through enhanced solubilization of nutrients, increased nutrient uptake facilitated by improved root growth, and the sequestration of nutrients (Gour & Purohit, 2004).

Induced systemic host resistance Induced resistance represents the most indirect type of antagonism, which can manifest locally or systemically. Systemic acquired resistance involves key components such as salicylic acid (SA) and non-expressor of pathogenesis-related genes1 (NPR1) (Deshmukh et al., 2006).

1.2 Aims and Objective

Aim- To isolate and characterize a potential biocontrol agent against foliar disease of *Solanum lycosperscium* (Tomato).

Objectives-

- Screening and isolation of potential biocontrol species.
- Characterization of the selected potential biocontrol species and determining the most effective isolate.
- Investigating the *in viv*o efficacy of the selected biocontrol agent via pot trials.

1.3 Hypotheses/Research question

The research question to be answered through this dissertation work is that “How can *Solanum lycospersicum* (Tomato) be protected from fungal pathogen like *Myrothecium* sp. causing foliar disease (Leaf spots) by means of a biological agent”.

1.4 Scope

Fungal diseases represent a significant challenge in various sectors, including agriculture, forestry, and human health. These diseases can infect crop, causing yield losses, reducing the quality of produce and sometimes complete crop failures. The economic impact of fungal diseases is substantial, with losses totaling billions of dollars annually worldwide. Increasing population has led to an increase in demand of vegetable crops like tomato. The use of chemical agents have shown to have negative effects on human, animal and plant health. It also has hostile repercussions on soil and also the environment. Hence finding a biological alternative is a must to reduce food wastage and increase production. Learning and discovering new biocontrol agents may prevent the spread of disease on not only tomato but also other commercial crops which face losses due to the same pathogen. Biological agents that produce plant growth promoting chemicals will also aid in increasing the overall harvest yield.

CHAPTER 2. REVIEW OF LITERATURE

Growing of plants in greenhouses has increased the occurrence of diseases in tomatoes as it provides suitable climatic conditions for foliar and stem disease to occur. Fungi enters through either the natural openings like stomata or while activities like pruning, harvesting etc. Some of the disease reported include early blight (caused by-*Alternaria solani*), late blight (caused by-*Phytophthora infestans*), septoria leaf spot (caused by-*Septoria lycopersici*), fusarium wilt (caused by-*Fusarium oxysporum f. sp. Lycopersici*), verticillium wilt (caused by-*Verticillium albo-atrum* and *Verticillium dahlia*), anthracnose (caused by-*Colletotrichum coccodes*), buckeye rot (caused by-*Phytophthora nicotianae var. parasitica*) and southern blight (caused by-*Sclerotium rolfsii*) (Sanoubar & Barbanti, 2017). Another foliar disease of tomato was observed in polythene covered greenhouse which spread rapidly the causal organism was found to be *Myrothecium rodium* Todd ex. causing myrothecium leaf spot (YanJie et al., 2009).

Myrothecium includes two plant pathogenic species known till now namely *Myrothecium rodium* and *Myrothecium verrucaria*. It has known to cause disease in vegetable plants(like tomato, cucumber, sweet pepper, lettuce, spinach),ornamental plants (like *Anthurium*, *Deffenbachia*) and solanaceous weed plants (like *Nicandra physaloides*) (Gilardi et al., 2018; Quezado Duval et al., 2010). It has also been reported in other commercial plants like coffee, cotton, *Vigna* varieties and mulberry (Maji et al., 2004; A. D. A. Silva et al., 2014; Taneja et al., 1990).

In an integrated management field trial, the combination of removing basal infected leaves and applying foliar sprays containing carbendazim WP or mancozeb WP demonstrated outstanding efficacy. This approach resulted in a disease severity of 6.5% for septoria leaf spot and a minimum disease severity of 1.6% for alternaria leaf spot (Anwar et al., 2017).

Studies have found that fungicides with tebuconazole, copper and quartenary ammonium were highly effective in inhibiting the mycelial growth under *in-vitro* conditions (Quezado Duval et al., 2010). Against root rot disease of tomato certain fungicides showed maximum inhibitory effect under *in-vitro* conditions they were - rizolex–T, moncut and maxim-XL followed by salicylic acid as chemical inducers (Aboelmagd, 2021).

The effectiveness of the fungicide mancozeb was evaluated by monitoring the percentage of disease intensity at 70, 90, and 105 days after transplanting tomato plants. The lowest disease intensity was observed in the treatment with fungicide mancozeb, with values of 15.43%, 17.90%, and 20.47% at 70, 90, and 105 days respectively. In comparison, the control group exhibited the highest disease intensity, with values of 25.50%, 33.47%, and 48.73% at the same respective time points. Hence proving Mancozeb to be a good chemical fungicide (Zghair, et al., 2014).

A study was carried out to find the effect of six widely used fungicide on soil health and its interactions with the soil enzymes. Majority of the fungicides were recorded to reduce the activity of soil enzymes while others manifested positive or controversial effects which was regulated not only by the chemical composition of fungicide but also by its quantity, time of exposure, and/or properties of the soil. The fungicide toxicity to soil enzymes was in the following order: mancozeb < carbendazim < azoxystrobin ≈ tebuconazole << chlorothalonil (Kenarova, & Boteva, 2023).

An investigation was conducted, employing three fungicides commonly found in the market: carbendazim, copper oxychloride, and mancozeb. Its aim was to assess their impact on soil fungi after application. The findings indicated a significant decline in microbial populations in the soil following fungicide treatments compared to the untreated control. This decrease in microorganisms resulted in diminished decomposition of dead leaf matter into organic

and inorganic components, ultimately reducing the incorporation of organic material into the soil structure and thereby impacting soil fertility (Ratna Kumar et al., 2018).

Studies have also shown that fungicides can have effects which can damage plant physiology and important plant functions like photosynthesis, reduction in net CO_2 assimilation, pigment biosynthesis inhibition, modifications in dark respirations etc. (Dias, 2012). Research indicates that fungicides modify the communities of microorganisms and fungi responsible for nutrient recycling in soil, while copper-based fungicides are harmful to earthworms and other fauna according to studies. Non-targeted animals and plants when exposed to such chemical agents can have adverse effects. When spraying of chemical agent occurs it gets settled on the skin/dermis layer of insects. These spray droplets consist of a concentrated active ingredient dissolved in either an oily or water-based carrier solution, sometimes supplemented with an adjuvant. When these small droplets come into contact with animals, they deliver a potent dose of toxin to their skin, hair, or feathers. Consequently, lipophilic insecticides are readily absorbed through the skin, leading to acute dermal toxicity that can often prove fatal to the animal (Sánchez-Bayo, 2012).

Thus to overcome such negative impacts of chemical agents occurring on soil health, plant and animals an alternative was conceptualized that are biocontrol agents. Hence different microorganism were used as an agent to stop the invasive/pathogenic species and help the plant thrive. The different biocontrols involve fungal, plant virus, arbuscular mycorhhizal fungi, yeasts, algal, cyanobacterial, phage based biocontrol. Some new emerging biocontrol stratergies involve microbial volatilome, microbiome based solutions, phage cocktail, genetically modified biocontrol agents, microbiome engineering etc. (Pandit et al., 2022).

Studies have found that bacillus species have shown antagonistic property against fungal diseases. One such study of *Bacillus velezensis* NKG-2 showed antagonistic properties

against major plant pathogens like *Fusarium oxysporum*, *Fusarium graminearum, Botrytis cinerea, Alternaria alternata, Fulvia fulva,* and *Ustilaginoidea virens*. It produced volatile organic compounds which affected the fungal growth. It also produced chitinase, cellulase, β glucanase etc. and it also increased plant production by producing indole acetic acid (IAA). In an in-vivo biocontrol experiment, tomato plants solely treated with pathogenic fungi exhibited a disease severity of 65%, whereas plants treated with the bacteria showed no symptoms, with disease severity only reaching about 25% at the time of sampling (Myo et al., 2019).

A study was conducted to check *Clonostachys rosea* LQC62 and *Trichoderma asperellum* LQC96 against *Myrothecium rodium* which acts as a pathogen in Begonia plant. It was found that it created volatile compounds which caused reduction of the pathogen under *in vitro* conditions. Foliar spray of LQ62 led to reduction of pathogen during commercial cultivation (Fujinawa et al., 2020).

In a conducted study, five Plant Growth Promoting Rhizobacteria (PGPR) from various genera were isolated from the roots of healthy tomato plants and characterized based on their ability to colonize roots and solubilize phosphate. Treatment with these bacteria resulted in a notable increase in seed germination, seedling vigor, tomato growth, and fruit weight. Additionally, seeds treated with these PGPR exhibited protection against *Alternaria solani*, likely due to the presence of hydrogen cyanide (HCN), siderophores and chitinase in the isolated PGPR. Moreover, significant accumulation of antioxidant enzymes such as polyphenol oxidase (PPO) and peroxidase (POX) was observed in plants pre-treated with PGPR, regardless of infection (Narendra Babu et al., 2015).

A study carried out found that plant growth promoting microbes *Bacillus subtilis* and *Pseudomonas fluorescens* have considerable scope in managing multiple fungal foliar disease and sustainably increase the yields of tomatoes (Hegde et al., 2022).

A study was conducted to investigate the potential of rhizobacteria to safeguard plants against naturally occurring diseases and enhance crop productivity. Rhizobacteria were introduced to crop plants through seed microbiolization technique. Different treatment groups were established, including various frequencies of fungicide application on tomato plants grown from microbiolized or non-microbiolized seeds over a period of 90 days. The control group consisted of non-microbiolized seeds without fungicide application. The study focused on foliar diseases such as early blight, septoria leaf spot, and late blight. All treatment groups exhibited reduced disease incidence and increased crop yields compared to the control. The findings suggested that employing a combination of rhizobacterial and chemical treatments in the field could potentially reduce the frequency of fungicidal spraying while simultaneously boosting crop yields (H. S. A. Silva et al., 2004).

Extensive research has highlighted the capacity of Burkholderia genera to produce diverse compounds that impede plant pathogens. A specific study identified that strains MTo293 and TTe203 of *Burkholderia tropica* exhibited the most substantial radial inhibition of mycelial growth. Morphological changes was observed in the pathogenic fungi (*Colletotrichum gloeosporioides, Fusarium culmorum, Fusarium oxysporum and Sclerotium rolffs*) like disruption of fungal hyphae. They also showed production of 18 volatile compounds involved in growth inhibition mechanism including α-pinene and limonene (Tenorio-Salgado et al., 2013).

A study was carried out to estimate the biocontrol potential of *Trichoderma* species. *Trichoderma harzianum* (ANR-1) isolate was found to effectively inhibit the radial mycelial

growth of the pathogen (by 53%) - *fusarium oxysporum f. sp. lycopersici* (FOL) causing fusarium wilt. The application of *Trichoderma harzianum* (ANR-1) under greenhouse conditions resulted in minimum disease incidence (by 15.33%). Also tomato plants treated with *Trichoderma harzianum* (ANR-1) exhibited a significant increase on plant height and it also showed increase in the dry weight of tomato plants in comparison to other isolates and untreated control (Sundaramoorthy & Balabaskar, 2013). The bioagents *Trichoderma harzianum* and *Pseudomonas fluorescens* have shown to have effect on reducing severity of the disease early blight caused by fungi in experimental field using seed treated and foliar spray technique (Zghair, et al., 2014).

Studies have showed use of endophytic fungi (fungi residing asymptomatically within plant) are a suitable candidate for biocontrol application. Different endophytic fungi such as *Colletotrichum, Cladosporium, Fusarium, Pestalotiopsis* and *Trichoderma* are an attractive option for management of some plant diseases and their ability were tested using in-vitro and in-vivo assays (De Silva et al., 2019).

A study was carried out to find the antagonistic activity of phyllosphere yeasts against infectivity of *Botrytis cinera* causing grey mould. The efficacy of yeast isolates in suppressing grey mould was assessed using a detached leaf assay. Nine yeast isolates demonstrated capability to reduce disease index by over 90% compared to an untreated control. In greenhouse trials, *Rhodotorula glutinis* Y-44 emerged as the most effective agent in controlling grey mould in tomato plants. Since *Botrytis cinerea* is a prominent post-harvest pathogen affecting tomato fruits, the potential of *R. glutinis* Y-44 to protect artificially wounded and infected tomato fruits was also examined. The findings concluded that *R. glutinis* Y-44 could reduce wound severity by 50% compared to untreated controls (Kalogiannis et al., 2006).

In Brazil, the control of late blight heavily relies on the use of chemical agents, prompting a study to explore biocontrol alternatives. A total of 23 rhizobacterial isolates and 208 epiphytic microorganisms were collected from organically cultivated tomato plants and conventional fields. These were then screened for their antagonistic activity against *Phytophthora infestans*, the causal organism of late blight. Based on in vitro assays measuring inhibition of sporangia germination and detached leaflet bioassays, four epiphytic microorganisms (*Aspergillus* sp., *Cellulomonas flavigena*, *Candida* sp., and *Cryptococcus* sp.) were identified as promising candidates. These microorganisms were subsequently applied individually or in combination on tomato plants, with or without prior treatment with the rhizobacteria *Bacillus cereus*. Control plants exhibited high rates of late blight progression (r), area under the disease progress curve, and final disease severity. The lowest values of disease severity were observed in plants colonized by *B. cereus* and treated with *C. flavigena*, *Candida* sp., and *Cryptococcus* sp. Interestingly, there was no reduction in disease severity in plants treated solely with rhizobacteria (Lourenço Júnior et al., 2006).

Study carried out reported that microbial consortia made of *Trichoderma asperllum* and *Pseudomonas fluorescens* showed disease reduction potential against late blight caused by *Phytopthora infestans* and also had plant promoting potential (Singh et al., 2022). A study found that a hypersaline bacteria, *Bacillus paralicheniformis* strain MPSK23 was effective as an succesfull biocontrol agent against fusarium wilt caused by *Fusarium solani* in Chilli plant belonging to solanaceace family (Pawaskar & Kerkar, 2023).

Research revealed that creating a microbial consortium by carefully selecting well-characterized beneficial bacteria and fungi with diverse biocontrol mechanisms results in a more adaptable biocontrol agent compared to single strains. This consortium exhibits enhanced functionality, as it can effectively control a broader spectrum of diseases. The study concluded that while different individual microorganisms were most effective against

specific pathogens like the root pathogen *Fusarium oxysporum* or the foliar pathogen *Botrytis cinerea* in tomatoes, the consortium displayed extended functionality. It efficiently controlled both pathogens regardless of the application method, consistently achieving the same level of protection as the most effective single strains (Minchev et al., 2021).

Utilizing biological control and hormonal inducers presents an intriguing strategy for inducing disease resistance against pathogens, particularly when used in combination. A study was conducted wherein tomato plants infected with *Fusarium oxysporum* were subjected to applications of hormonal inducers such as JA (Jasmonic Acid) and SA (Salicylic Acid) while being inoculated with arbuscular mycorrhizal (AM) fungi. The results indicated a gradual increase in the percentage of disease incidence in infected plants over time, reaching 86% at 42 days. However, treatments involving AM fungi, JA, and SA significantly reduced the percentage of disease incidence. The combination of AM fungi and JA demonstrated the highest effectiveness, achieving 92% efficiency. Moreover, the growth rate (both shoot and root) and the percentage of mycorrhiza colonization were markedly inhibited in tomato plants in response to fusarium wilt disease compared to healthy controls. However, the application of AM fungi, JA, and SA, especially when combined, exhibited pronounced effects in enhancing tomato growth (Khallal, 2007).

A study was conducted to evaluate the effectiveness of combining biocontrol agents with essential plant nutrients, such as nitrogen, phosphorus, potassium (NPK), and zinc (Zn). The objective was to assess the integrated impact of *Bacillus subtilis* (BS-01) and these selective plant nutrients against the foliar disease early blight and the overall response of the crop. Results from the field study indicated that the combined effect of BS-01 with plant nutrients significantly enhanced the resilience of infected tomato plants against early blight. This was achieved by effectively altering the levels of total chlorophyll, carotenoids, and total phenolics, as well as the activities of antioxidant enzymes like PPO, SOD etc. Furthermore,

this treatment was notably effective in reducing the pathogen load in tomato foliage, demonstrating a desirable level of defense against infection caused by *A. solani*. Additionally, the interaction of BS-01 + Zn + NPK synergistically contributed to enhancing crop productivity, resulting in the highest marketable yields (Awan et al., 2022).

New emerging biocontrol strategies have been discovered which include using of “plant optimized microbiomes” (microbiome engineering) and establishing the genetic foundation of advantageous interactions between plants and microbes to facilitate the development of crops optimized through microbial breeding. This will help is improving, facilitating and maintaining long term colonization as compared to simply applying individual biocontrol microbes (Syed Ab Rahman et al., 2018).

CHAPTER 3. METHODOLOGY

3.1 Isolation and characterization of pathogen from infected tomato plant

3.1.1 Sampling and isolation of pathogenic fungi

Tomato plants infected with fungal pathogens showing visible symptoms on leaves like spots were selected for this experiment. Such infected plant sample was collected and packed in plastic bags and then transferred to the laboratory. The infected leaf samples were first thoroughly washed with distilled water and then the underlying fungi was isolated from the infected plant tissue using sterile scalpel and forceps. The infected plant tissue sample, with a small surrounding portion of uninfected tissue, was cut into small pieces measuring 2-5mm squares. Using flame-sterilized forceps, these tissue pieces were then transferred to sterile petri dishes containing a 1% sodium hypochlorite solution for 1-2 minutes to surface sterilize the plant tissues. Subsequently, the infected leaf tissue was washed three times in sterile distilled water and dried between two sterile filter papers. The dried infected plant parts were then placed onto petri plates of Potato Dextrose Agar (PDA) medium supplemented with Chloramphenicol and incubated at $28 \pm 2^{o}C$ for 3-4 days to let the fungi grow completely. Using the hyphal tip technique the resulting fungi were then purified on PDA medium, and each isolated fungus was sub-cultured on slant medium for future studies (Amani & Avagyan, 2014; Thilagam et al., 2018).

3.1.2 Tentative identification of pathogenic fungi

The identification of fungi was carried out with help of direct mount from pure culture. For the identification different characteristics were taken into consideration. Colony characters such as growth, pigmentation, texture, colour of colony on front and reverse side of petri plate, diameter of colony etc. Cultural characteristic like conidial heads, conidiophores, conidiogenous cells and conidia were considered for identification. The microscopic structures were visualized by preparation of slides using lactophenol cotton blue dye and observed under low and high power magnifications. The purified fungi were verified identified using references from (Nagamani et al., 2006).

3.1.3 Pathogenicity testing

The isolated fungal pathogen's pathogenicity was assessed by incubating it on Potato Dextrose Agar (PDA) for 20 days at 28$\pm$ 2^{o}C. Conidia of fungal pathogen were harvested from PDA by adding 5-10mL sterile distilled water and gently swirling the plate to dislodge the conidia. The concentrated conidial suspension was adjusted to a concentration of 10^{6} conidia/mL and served as the inoculum (Than et al., 2008). The wound/drop method was employed to inoculate tomato leaves with the prepared conidial suspension. The wound created on the leaf surface was 6-9 mm long. As control the tomato leaf was inoculated with sterile distilled water. After lesion formation a piece of the infected tissue was cut, surface sterilized using 1% Sodium hypochlorite and inoculated in a plate containing PDA to check whether the inoculated fungus could be re-isolated and to prove Koch postulates (Kwon et al., 2013).

3.2 Screening and isolation of potential biocontrol species.

3.2.1 Collection of Soil sample

Rhizospheric soil of healthy tomato plant was collected and packed in plastic bags from both field and home garden. The soil sample was stored in the refrigerator till the sample was further processed.

3.2.2 Serial dilution and spread plating

Serial dilution was conducted by adding 1g of soil sample to 9ml of sterilized distilled water, followed by shaking for 30 minutes before spread plating. Suspension was serially diluted until 10^{-5} dilution with water. Dilutions 10^{-3}, 10^{-4} and 10^{-5} were spread plated on petriplate containing nutrient agar medium with amphotericin which is an antifungal supplement to supress fungal growth .Incubation was carried out at $28\pm2^{o}C$ for 2 days. Single colonies with distinct morphologies were chosen, isolated, and purified through repeated streaking until pure isolates were obtained (Adal & Lemma, 2023).

3.2.3 Screening of isolates for biocontrol potential using dual culture inhibition test

Bacterial isolates were spot inoculated equidistantly along the margins of Potato Dextrose Agar (PDA) medium containing petri plates and incubated for 48 hours at 28 ± 2°C. Fungal plant pathogens from PDA cultures were then placed at the center of each bacterial isolate-grown plate and incubated for 10-15 days at 28 ± 2°C. As control, plates containing fungal discs without test bacteria were included. The inhibition of fungal growth by bacterial isolates was evaluated by observing the presence of inhibition zones on the dual culture

plates. The degree of inhibition was assessed by measuring the percentage radial growth inhibition zone (PIRG) using the formula

PIRG = (C-T)/C * 100

where PIRG represents the percentage inhibition of radial growth, C is the radial growth measurement of the pathogen in the control, and T is the radial growth of the pathogen in the presence of antagonistic bacteria (Landa et al., 1997). The isolates that showed better inhibition were selected for further biocontrol tests and plant growth promotion.

3.3 Characterization of the selected potential biocontrol species and determining the most effective isolate

3.3.1 Colony morphology

The selected biocontrol species were characterized based on colony morphology by growing them on nutrient agar and observing their color, elevation, surface, shape, and pigmentation features, which were then documented in tabular form (Adal & Lemma, 2023).

3.3.2 Gram reaction

A loopful of water was placed on clean glass slide .Colony were touched with loop and mixed thoroughly with water to form thin film of microorganisms on the slide. Sample were then air dried and heat fixed. Crystal violet stain was used to stain the slide for one minute, followed by rinsing with water. Subsequently, iodine solution was applied to the slides for one minute and then rinsed off. The slides were treated with Gram's stain decolorizer for 30 seconds, followed by rinsing with water. They were then counterstained with the help of safranin for about 30 seconds, rinsed with water, and left to dry. Slides were visualised under oil immersion objective of microscope (Tripathi & Sapra, 2024).

3.3.3 Production of hydrolytic enzymes and bioactive compounds

3.3.3.a Chitinase test- Bacterial isolates were examined for chitinase production by cultivating them on colloidal chitin agar (CCA) medium. The CCA medium was produced by adding 5 g of colloidal chitin along with mineral salts (KH_2PO_4 0.7 g, K_2HPO_4 0.3 g, $MgSO_4 \cdot 5H_2O$ 0.5 g, $FeSO_4 \cdot 7H_2O$ 0.001 g, $ZnSO_4$ 0.001 g, $MnCl_2$ 0.001 g, and agar 20 g for 1 L with pH 8) (Sasi et al., 2020). Formation of halo zone around bacterial colony post flooding it with grams iodine solution indicated chitinolytic activity.

3.3.3.b Cellulase test- Cellulase production in bacterial isolates was evaluated by inoculating them onto M9 agar medium containing 10 g/l carboxymethyl cellulose (CMC). After 48 hours of incubation at 28$\pm$2°C, the plates were flooded with congo red dye. The presence of clear halos surrounding the colonies indicated their cellulolytic activity (Bhattacharyya et al., 2020).

3.3.3.c Protease activity- Protease activity of the bacterial isolates was assessed by inoculating them onto skim milk agar (skim milk powder 10 g l^{-1}, agar 15 g l^{-1}). Protease activity was demonstrated by the formation of clear zones around the colonies (Adal & Lemma, 2023).

3.3.3.d Hydrogen cyanide (HCN production)- HCN production was assessed for all isolates by streaking them on slants containing nutrient agar (NA) media along with filter paper strips soaked in solution containing picric acid and 2% sodium carbonate. The test tubes were sealed with parafilm and then incubated at 28±2°C for 3–5 days. The change in color of the yellow filter paper strips to brown or red indicated HCN production

3.3.3.e Ammonia production- Ammonia production was assesed for each bacterial isolate by culturing them in peptone broth (10 mL) and incubating at 28±2°C for 2-3 days. After incubation, Nessler's reagent (0.5 mL) was added to the bacterial suspension. The production of ammonia was detected by observing a color change from yellow to brown (Lorck, 1948).

3.3.3.f Lipase production- Tributyrin agar media was used for detection of bacteria which could produce lipase. 23 gms of tributryin agar base was added in 990ml of distilled water, to this 10 mL of Tributyrin was added and heated to boiling to dissolve the media and then autoclaved. Lipase activity was demonstrated as clear zones around colony (Samad et al., 1989).

3.3.3.g Siderophore production- The rhizosphere associated bacterial isolates was screened for siderophore production using solid medium containing Chrome Azurol Sulfonate (CAS agar). It was prepared by adding protease peptone - 20g/l, Dipotassium Hydrogen Phosphate-1.5g/l, $MgSO_4.7H_2O$-1.5g/l, Agar-20g/l and pH adjusted to 7.2. The cas dye was prepared separately by adding 18.51mg Chrome Azurd dye in 4ml distilled water, 21.87mg CTAB in 3ml distilled water and 16.22mg $FeCl_3$ in 3ml. This three solution were then mixed autoclaved separately and then added in the media before pouring. The reaction was considered positive when an orange halo surrounding the bacterial colony appeared due to the removal of iron from CAS by the siderophore (Bhattacharyya et al., 2020).

3.3.4 Plant growth promotion activity

3.3.4.a Phosphate solubilising test- The phosphate solubilization test involved inoculating each isolate onto Pikovskaya agar medium with the pH adjusted to 7.0. The plates were then incubated at 28±2°C for 3–5 days. The growth and presence of clear zones around the colonies were examined to determine phosphate solubilization. Isolates that exhibited a clear halo zone surrounding them were identified as solubilizers of tricalcium phosphate (TCP) (Landa et al., 1997).

3.3.4.b Nitrogen fixtation test- The nitrogen fixation test was conducted using nitrogen-free media, specifically Jensen's nitrogen-free medium. A qualitative evaluation of nitrogen fixation was performed by culturing the bacterial isolates for 5 days at 28±2°C. A positive test for nitrogen fixation was indicated by the production of a pellicle at the subsurface level (Adal & Lemma, 2023).

3.3.4.c Indole acetic acid (IAA) production- The production of indole-3-acetic acid (IAA) by the bacterial isolates was assessed by culturing bacterial cultures in LB (Luria-Bertani) medium supplemented with 1-tryptophan (1.02 g/l) and incubating them at 28±2°C for 3–5 days. After incubation, centrifugation of the bacterial cultures were carried out at 7000 rpm for 3 minutes, and 1ml of the supernatant was mixed with 2ml of Salkowski reagent (containing 60% perchloric acid and 3 ml of 0.5 M FeCl3 solution). The development of a pink coloration upon addition of Salkowski reagent was an indication for IAA production (Laskar & Sharma, 2018).

3.4 Biochemical identification of selected biocontrol agent

3.4.1 Motility test- Motility of the selected isolate was tested through hanging drop method and SIM media

Hanging drop method- A small amount of bacteria was positioned in the middle of a coverslip. The glass side was positioned over the coverslip so that it adhered to the gel at the coverslip's corner. The glass slide was then lifted and flipped over. The droplet was examined using the low-power (10x) dry objective of the compound microscope, with attention given to the periphery of the droplet. Once located, the edge of the drop was observed under the 40x high-power objective to assess bacterial movement.

Motility testing using SIM media- Single colony of the bacteria was inoculated into sulphide indole and motility (SIM) media in a test tube. The test tube was then incubated for 48 hours at 28±2°C. The assessment of the motility test entailed observing the bacterial growth on the medium. Bacteria that solely proliferated around the point of insertion signified a negative outcome, whereas bacteria that dispersed across the surface or within the entirety of the medium indicated a positive result.

3.4.2 Catalase test- The catalase test involved the addition of 3% hydrogen peroxide (H_2O_2) to cultures that were 24 hours old. A positive outcome in the catalase test was defined by the emergence of oxygen bubbles, denoting the production of the catalase enzyme by the bacteria. This enzyme facilitates the conversion of hydrogen peroxide (H_2O_2) into water (H_2O) and oxygen (O_2).

3.4.3 Oxidase test- Oxidase test was carried out by adding loopful of bacteria on to the oxidase disc. Change of the disc colour from white to blue indicates positive results (Ismail et al., 2018).

3.4.4 IMViC test- IMViC tests were carried out using the HiMedia's Raped biochemical identification test kits-HiIMViC. 50ul of bacterial culture was to each test well and incubated for 24 hours followed by addition of the reagents provided.

3.4.5 Sugar utilisation test- Sugar utilisation by the bacteria was studied using HiMedia's – HiCarbo Kit (KB009A / KB009B1 / KB009C). 50ul of bacterial suspension was added to each test well and the results were checked after 24 hours incubation.

3.4.6 Molecular characterisation of selected bacteria.

For molecular identification the pure culture of bacteria were streaked on plates and slants. The identification was carried out using 16S rRNA gene.

3.5. Conduction of pot trials to test the *in vivo* efficacy of the selected biocontrol agent.

Pot trials were carried out in plastic pots. Four sets (biocontrol agent-test, chemical control-positive control, negative control, uninfected-untreated control) of treatments were prepared in duplicates. Soil was obtained from agricultural fields and autoclaved. Appropriate quantity of soil as per capacity of pots were added. Healthy tomato seeds were surface disinfected using 1% Sodium hypochlorite and thoroughly rinsed with sterile distilled water and then used for the experiment (Sharma et al., 2023).The seeds were sowed and allowed to germinate to form saplings. The saplings were then transplanted into four different sets of pots.

The selected biocontrol agent was grown in nutrient growth medium for 48-36 hours. The medium was centrifuged, the pelleted cells were then suspended in sterile distilled water. This biocontrol suspension was made of concentration of 10^6 bacterial cells/mL. For positive control chemical agent Bendaco (carbendazim-12%w/w+mancozeb-63%ww) was used by adding 2gm of it in 1 litre of sterile distilled water. Negative control sterile distilled water was used. All four (test, positive control, negative control, untreated-uninfected control) were sprayed on the tomato leaves a day prior to setting of infection.

With the help of sterile blade, small wounds of size 2mm x 1mm were created on tomato leaf surface. In this wound spore suspension of pathogenic fungi (inoculum) were added. Spore suspension was made by incubating the fungal culture on PDA for 15 days at room temperature. Conidia of fungal pathogen were harvested from PDA by adding 5-10mL sterile distilled water and gently swirling the plate to dislodge the conidia. The contained conidial suspension was adjusted to 10^6 conidia/mL and was used as the inoculum (Kwon et al., 2013). The disease development was noted every two days.

CHAPTER 4. ANALYSIS AND CONCLUSIONS

4.1 Identification of Plant pathogen

Diseased tomato leaves were identified based on morphology of the spots. The spots appeared to be brown coloured, circular with yellowing around the spots. A total of 12 Fungal cultures (SM01-SM12) were isolated from the leaves of which one culture was identified to be phytopathogenic – SM02. SM02 was identified to be *Myrothecium* sp. according to "The handbook of soil fungi by Nagamani et al. 2006" by studying the cultural properties, morphological and microscopic characteristic of the fungus. *Myrothecium* sp. is known to cause myrothecium leaf spot in tomatoes.

Fig 1. a) Healthy tomato leaves b) Diseased tomato leaves

Fig 2. Leaves showing disease spots

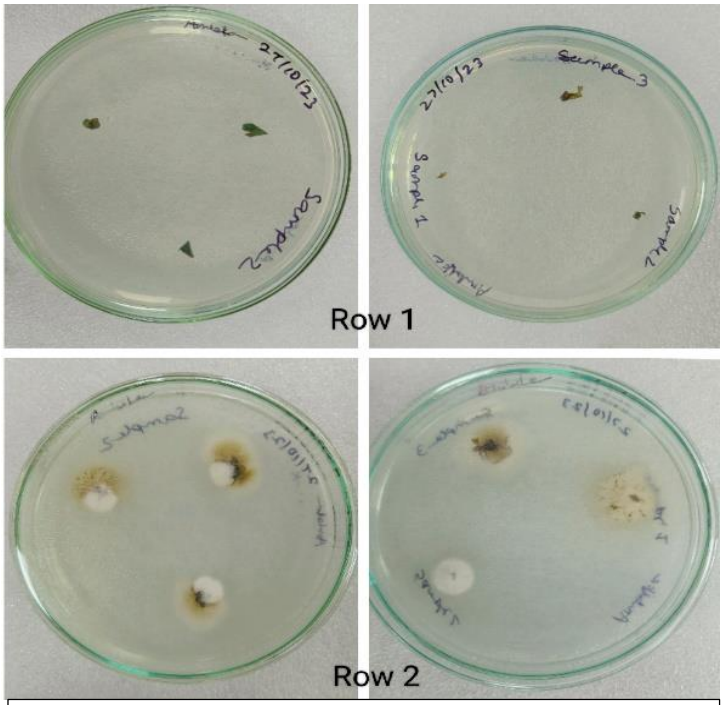

Fig 3. Row 1-Diseased plant tissue inoculated on PDA

Row 2- Growth of fungi observed after 2 days

4.2 Characterization of Plant pathogen

Taxonomy of SM02-*Myrothecium* sp.

- Kingdom-Fungi
- Phylum-Ascomycota
- Class-Sordariomycetes
- Order-Hypocreales
- Family-Stachybotryaceae
- Genus-*Myrothecium*

Colonies on inoculation on Potato Dextrose Agar showed abundant white mycelia and dark sporodochia distributed in concentric rings, reverse yellow coloured. Slow growing, attaining a diameter of 7.5 cm in 25 days. Under a 40X objective, SM02 exhibited conidiophores characterized as macronematous, densely clustered to form sporodochia, and branching extensively. The terminal branches of these conidiophores bear conidiogenous cells arranged in whorls, appearing hyaline and olivaceous. The conidiogenous cells are monophialidic, individually distinct, and cylindrical in shape.

Fig4. SM02 isolate on PDA (front)

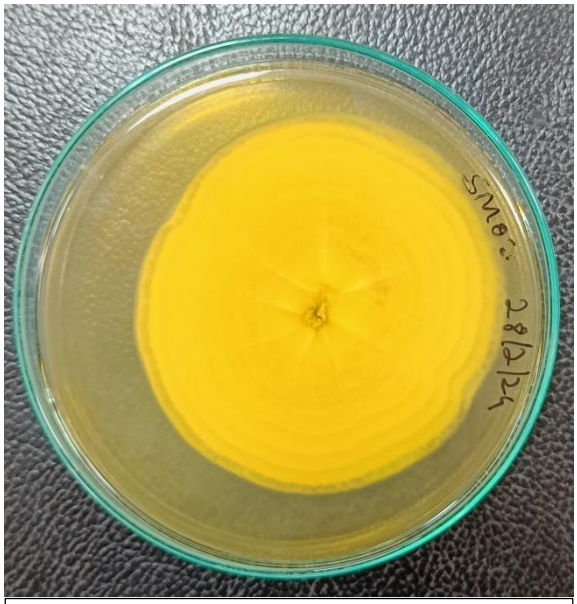

Fig5. SM02 isolate on PDA (reverse)

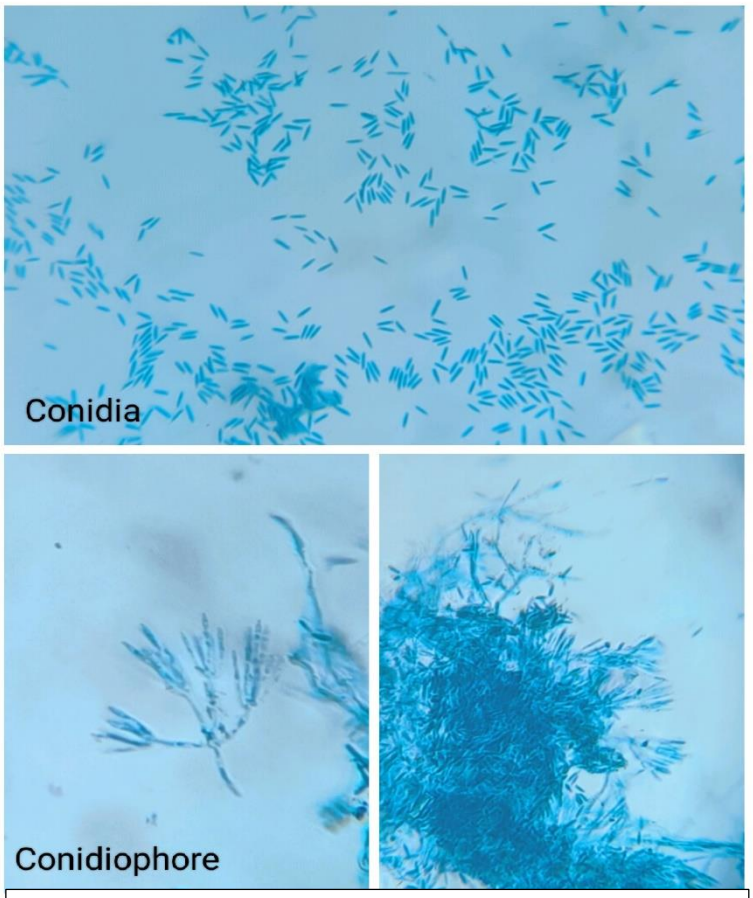

Fig 6. Microscopic analysis of SM02

4.3 Pathogenicity test of SM02

Post inoculation of SM02 suspension in the cuts made on leaf, lesion formation was observed within 5 days. The lesion formed was brown in colour with yellowing around it thus showing signs of infection. When placed on PDA plate the lesion formed portion led to re-isolation of *Myrothecium* sp.

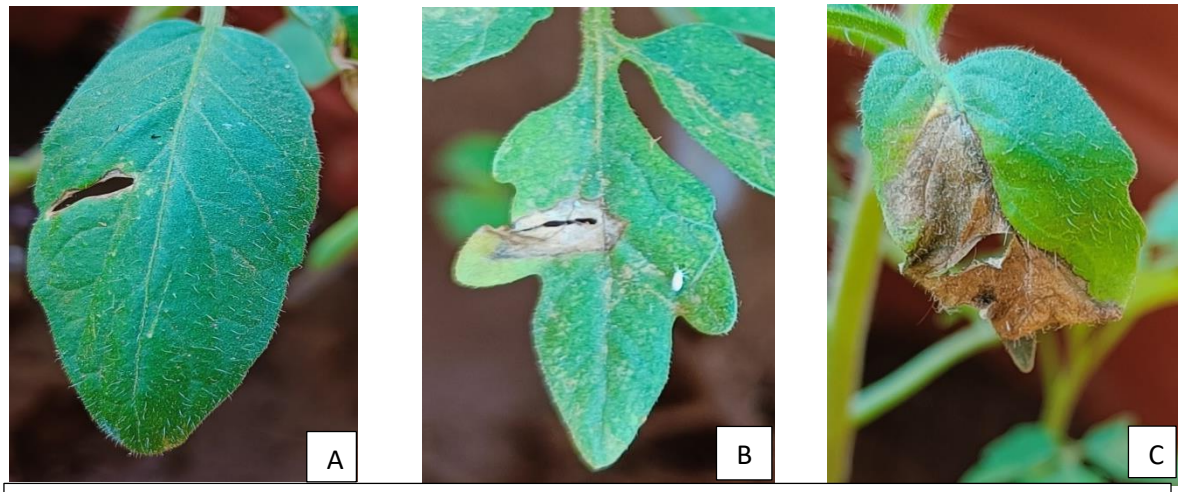

Fig 7. Symptom development after inoculation of *Myrothecium* sp. on tomato leaves.

A-Scars on the leaf were inoculated with sterile water (Control).

B-Scars in leaf inoculated by *Myrothecium* sp. and show disease symptom of brown and yellow tissue.

C- Scars in leaf inoculated by *Myrothecium* sp. and show disease symptom of brown and yellow tissue

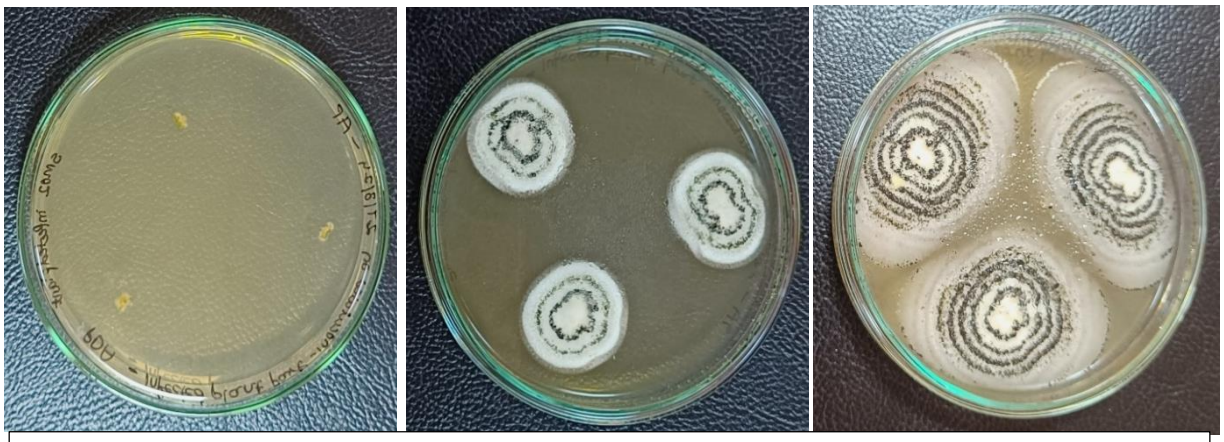

Fig 8. Re-isolation of same fungi (SM02-*Myrothecium* sp.) from infected plant tissue.

4.4 Screening and Isolation of Potential Biocontrol Agents

Following serial dilution and three days of incubation, a total of 42 bacterial isolates (RSSM01-RSSM42) were obtained from the rhizospheric soil of potted plants and fields, exhibiting variations in shape, texture, margin, pigmentation, and elevation.

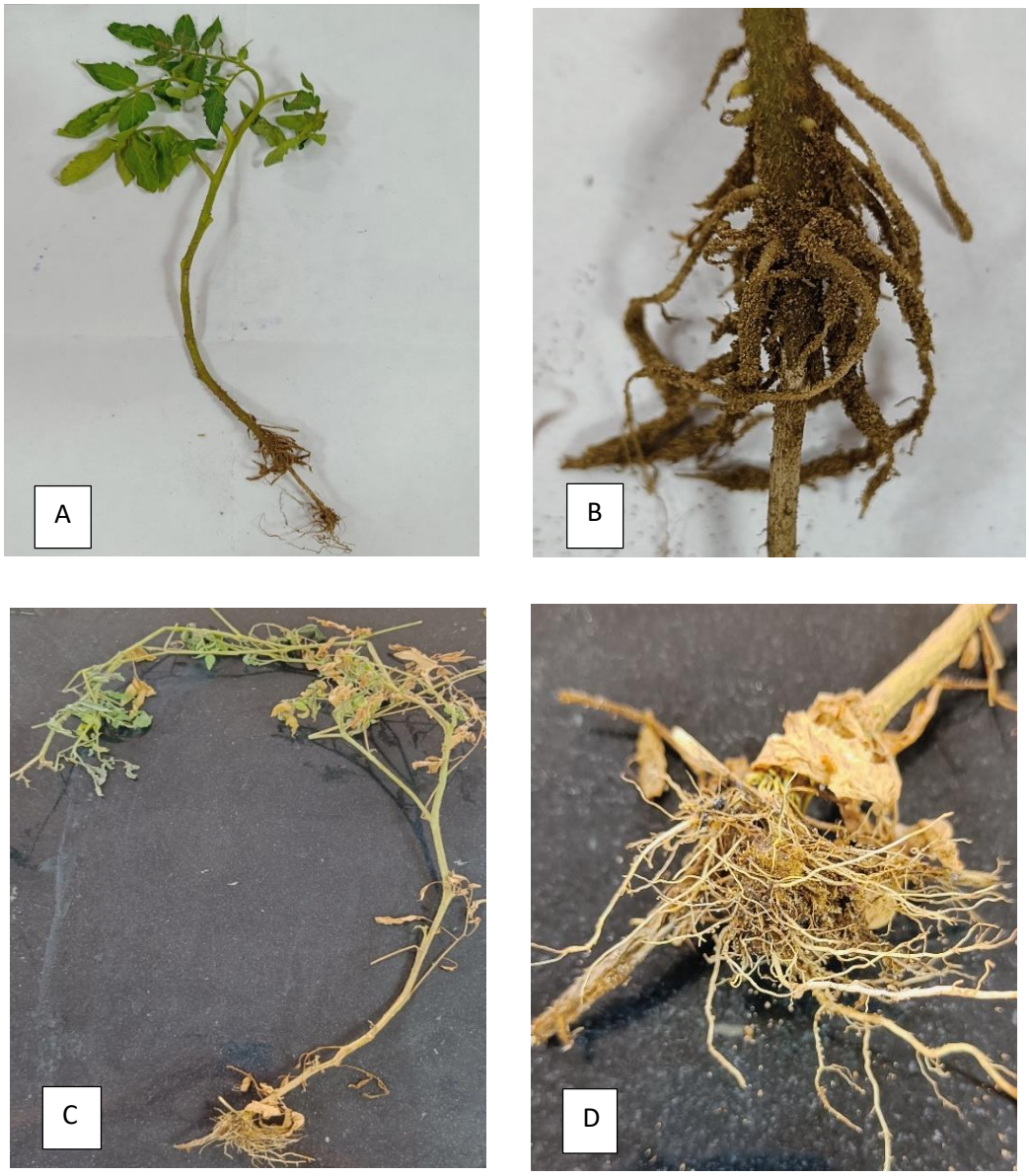

Fig 9. Healthy tomato plant and rhizospheric soil attached to it root.

A- Healthy tomato plant from field , B- Closeup image of rhizospheric soil attached to A

C- Healthy tomato plant from pot , D- Closeup image of rhizospheric soil attached to C

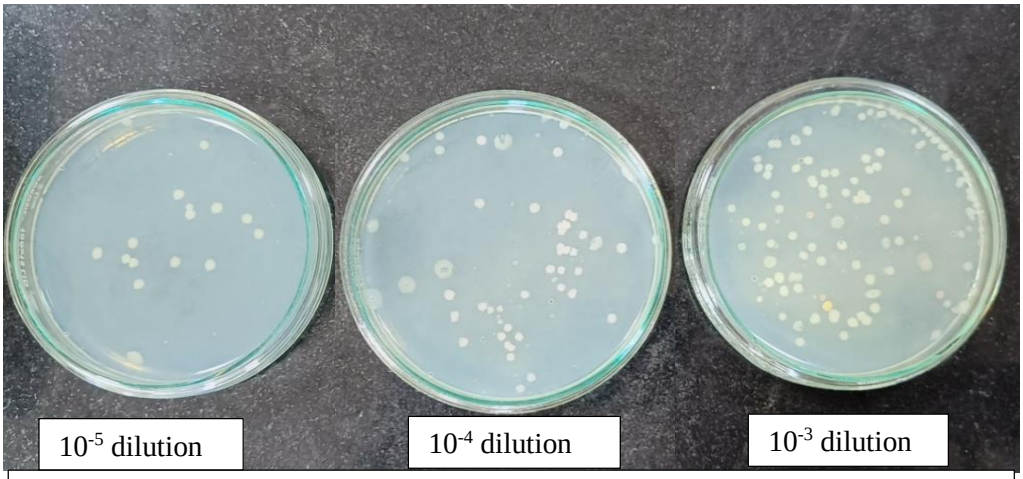

Fig 10. Serially diluted plates showing different colonies

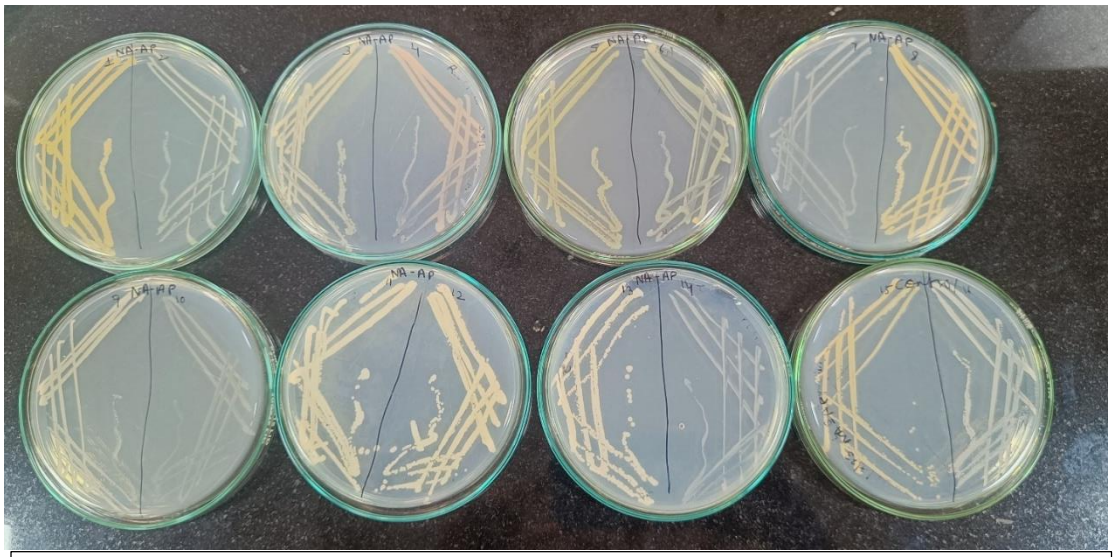

Fig 11. Purified rhizospehre associated bacterial colonies

4.5 Screening the bacterial isolates against *Myrothecium* sp.- Dual culture inhibition test

These isolates were screened against the fungal pathogen *Myrothecium* sp. using a dual culture inhibition test. Among the 42 rhizosphere associated bacterial isolates, 5 (11.9%) of them demonstrated inhibition of *Myrothecium* sp. growth. The isolates exhibited fungal radial growth inhibition reaching from 31.34% to 50.68%, with the maximum (50.68%) and minimum (31.34%) inhibition observed in isolates RSSM13 and RSSM39, respectively. The antagonistic rhizosphere-associated bacterial isolates significantly restricted the radial growth of the fungal pathogen compared to the control treatment.

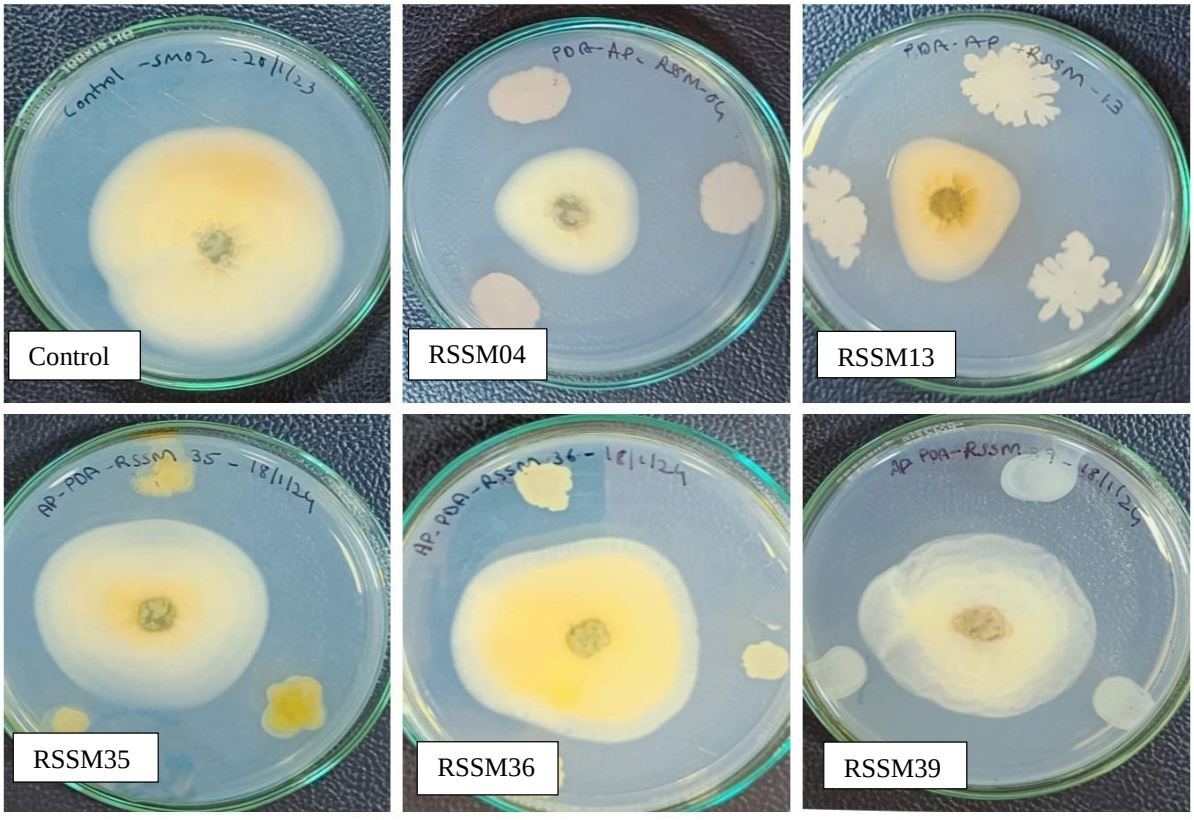

Fig 12. Rhizospehre associated bacterial isolates inhibiting pathogen *Myrothecium* sp.

Table 01: *Myrothecium* sp. inhibition by selected rhizospehre associated bacterial isolates

Isolate code	Source	Average inhibition in mm	PIRG %
RSSM 04	Rhizospheric soil of potted plant	31.35	43.36
RSSM13	Rhizospheric soil of potted plant	36.4	50.68
RSSM35	Rhizospheric soil of field plant	23.65	32.79
RSSM36	Rhizospheric soil of field plant	23.45	32.37
RSSM39	Rhizospheric soil of field plant	22.65	31.34

PIRG- Percentage Inhibition of Radial Growth

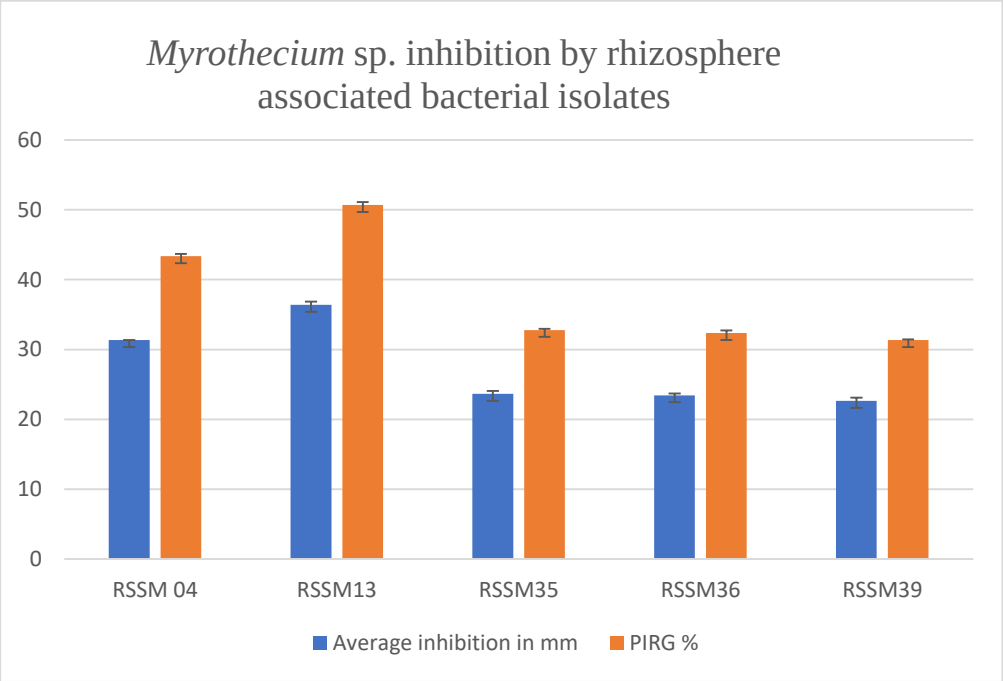

Fig 13. *Myrothecium* sp. inhibition by rhizosphere associated bacterial isolate

4.6 Characterization of selected Biocontrol agents.

The selected biocontrol isolates showed wide variation in colony morphology (Table 02).Regarding morphological properties of isolate 3 showed entire margin, 1 curled and 1 undulate. There were 3isolates with flat elevation, 1 crateriform and 1 raised. The pigmentation of colonies varied largely 2 were off white, 1 yellow 1 light pink and 1 red. All colonies were opaque .4 isolates had circular form and 1 irregular. All isolates were found to be Gram negative.

Table 02- Morphological and cultural characteristic of rhizosphere associated bacterial isolate

Isolates	Margin	Pigmentation	Elevation	Opacity	Form	Gram reaction
RSSM04	Curled	Red	Flat	Opaque	Circular	Negative
RSSM13	Undulate	Light pink	Crateriform	Opaque	Irregular	Negative
RSSM35	Entire	Yellow	Flat	Opaque	Circular	Negative
RSSM36	Entire	Off-white	Flat	Opaque	Circular	Negative
RSSM39	Entire	Off-white	Raised	Opaque	Circular	Negative

Fig 14. RSSM04

Fig 15. RSSM13

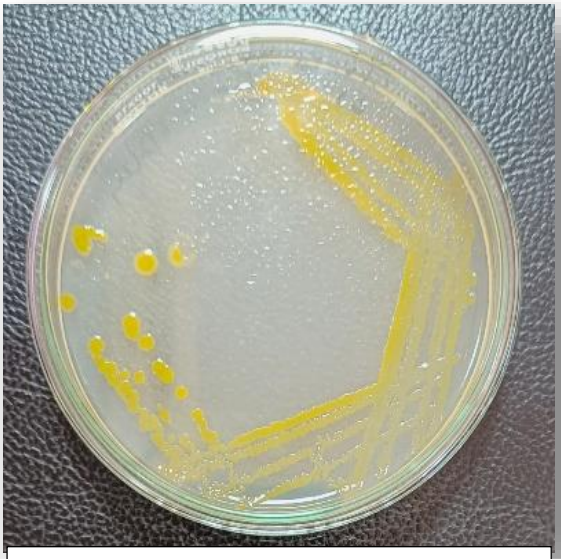

Fig 16. RSSM35

Fig 17. RSSM36

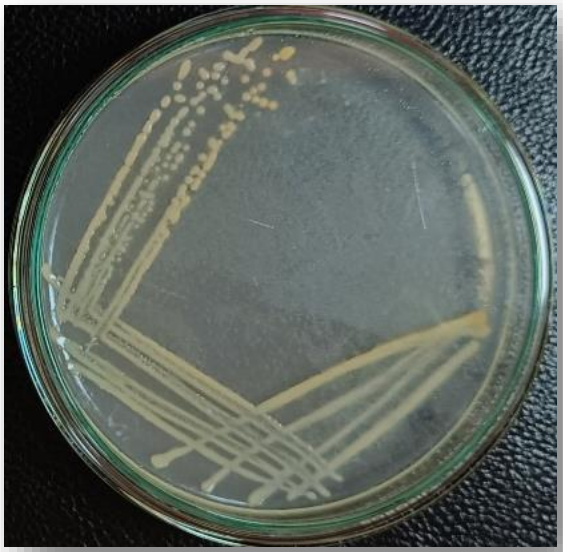

Fig 18. RSSM39

4.7 Screening for Hydrolytic enzymes and bioactive compounds

The potential of the selected rhizosphere associated bacterial isolates to produce hydrolytic enzymes and bioactive compounds are presented in that table (Table 03). All 5 isolates produced lipase, 4 of the isolates produced Ammonia, 3 of the isolates produced siderophore, protease, cellulase, 2 produced chitinase and only one isolate produced Hydrogen cyanide. The maximum biocontrol property was shown by RSSM04, RSSM13 and minimum was shown by RSSM36.

Table 03 Evaluation of isolates for biocontrol traits

	Isolate code				
Biocontrol traits	**RSSM04**	**RSSM13**	**RSSM35**	**RSSM36**	**RSSM39**
Chitinase	+	+	-	-	-
Cellulase	+	+	+	-	-
Protease	+	+	-	-	+
Lipase	+	+	+	+	+
HCN	-	-	+	-	-
Ammonia	+	+	+	-	+
Siderophore	+	+	+	+	-

+ = positive result

- = negative result

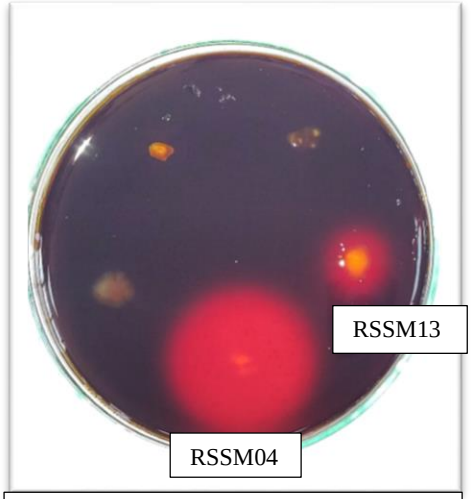

Fig 19. Chitinase Production

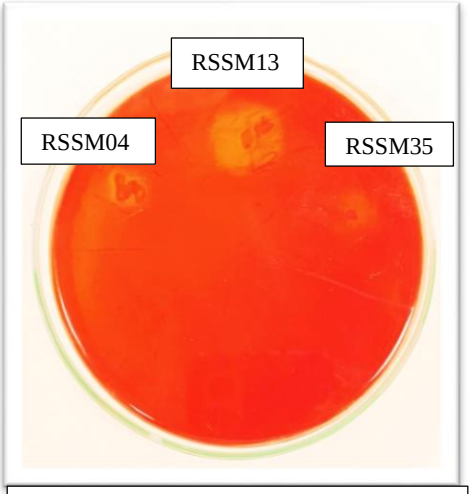

Fig 20. Cellulase Production

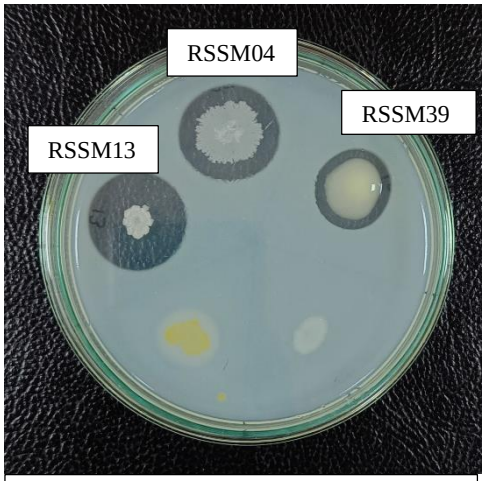

Fig 21. Protease Production

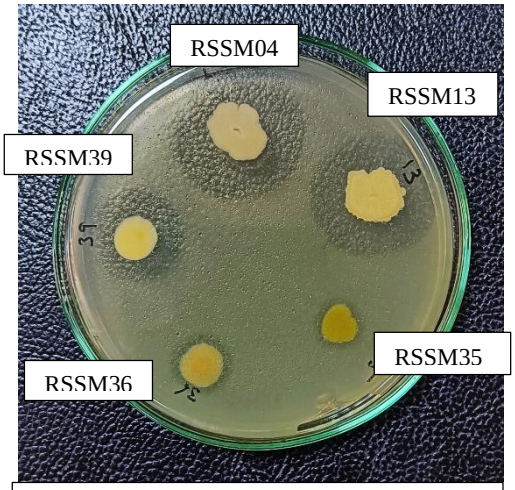

Fig 22. Lipase Production

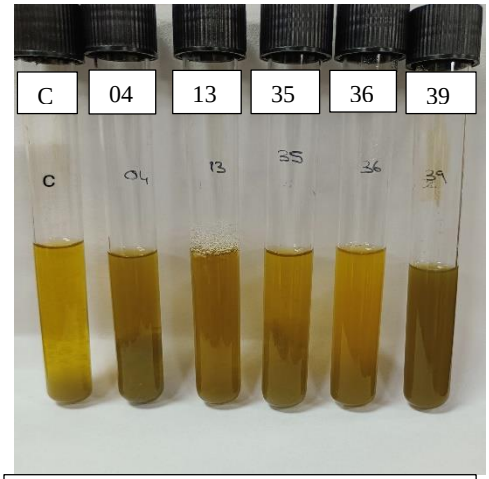

Fig 23. Ammonia Production

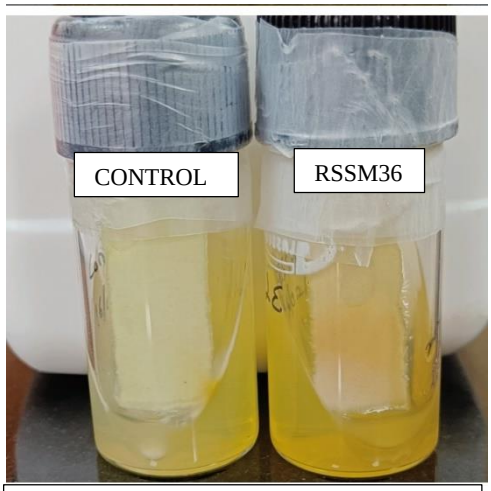

Fig 24. HCN Production

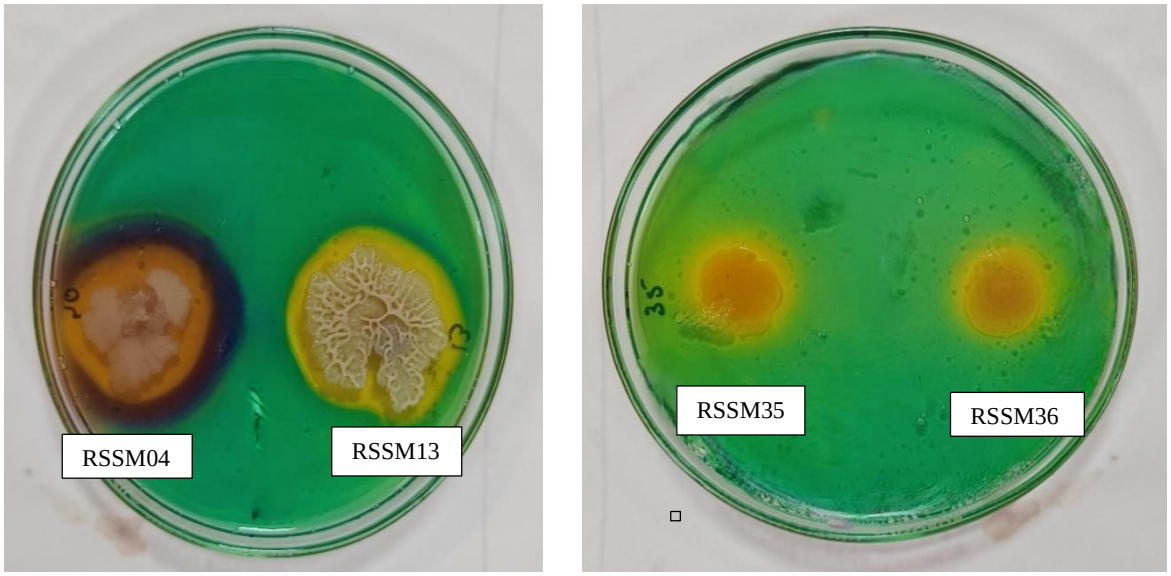

Fig 25. Siderophore Production

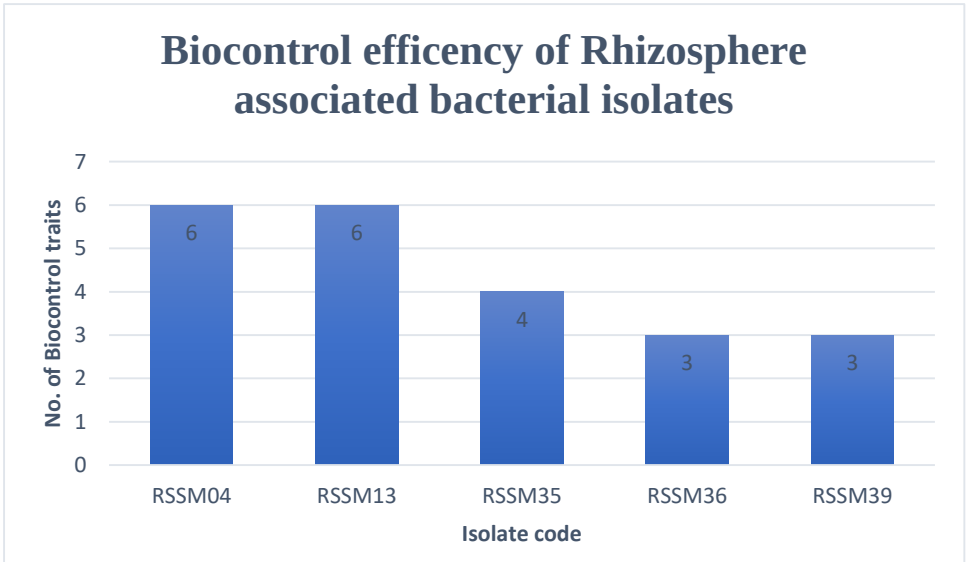

Fig 26. Selected bacterial isolates showing biocontrol traits.

4.8 Evaluation of the selected rhizospehre associated bacterial isolates for plant promotion traits

All the isolates tested positive for nitrogen fixation. Three of the isolates were indole acid producers and only one isolate tested positive for phosphate solublisation as seen in table below (Table04).

Table 04-Evaluation of Plant growth promotion traits of rhizosphere associated bacterial isolates

	Plant growth promotion traits		
Isolate code	**Phosphate Solublisation**	**Nitrogen fixation**	**Indole acid production**
RSSM04	-	-	-
RSSM13	-	+	-
RSSM35	+	+	+
RSSM36	-	+	+
RSSM39	-	+	+

+ = positive result

- = negative result

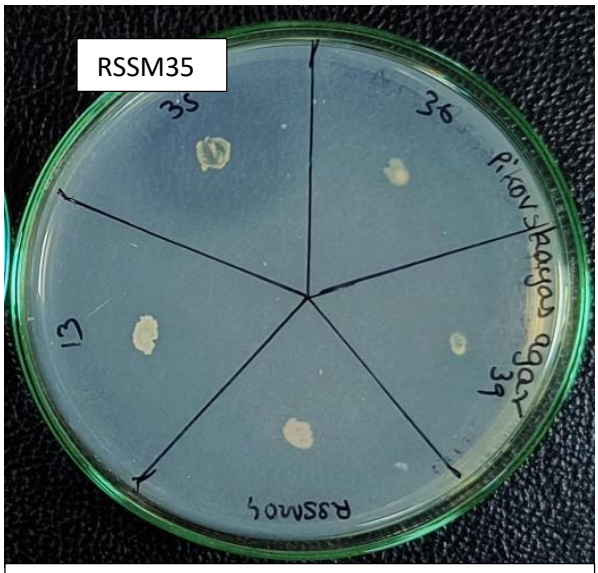

Fig 27. Phosphate solublisation

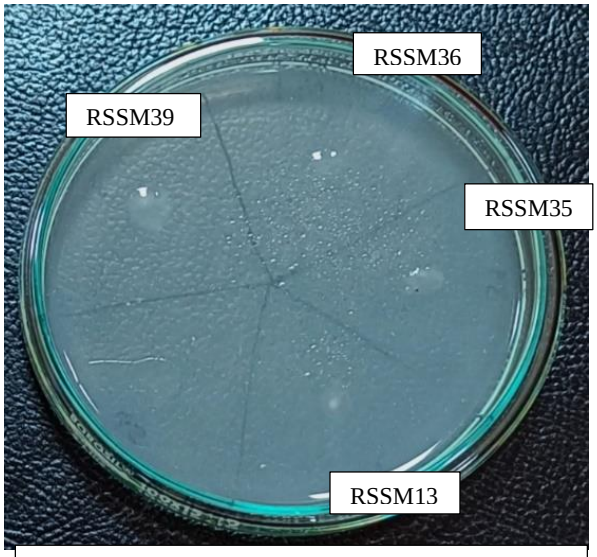

Fig 28. Nitrogen fixation

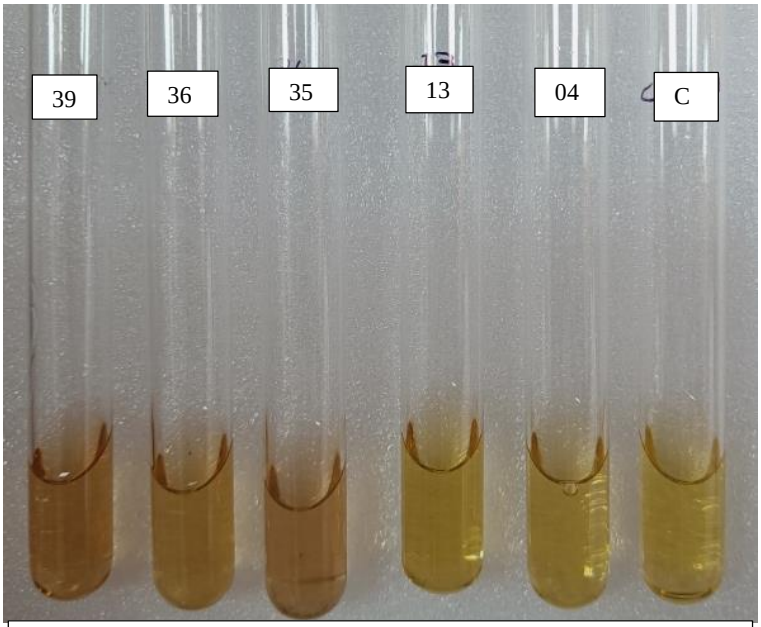

Fig 29. Indole acid production

4.9 Selection of final rhizosphere associated bacterial isolate and its biochemical identification

Taking into account the average inhibition, PIGR and biocontrol traits the isolate **RSSM13** was selected. RSSM 13 is a gram negative rod shaped motile bacteria. It has catalase and oxidase activity and can utilise the sugars- glucose, mannitol, maltose, fructose, dextrose, salicin, sorbitol, cellobiose, L-arabinose and sucrose.

Table 05 Biochemical tests of isolate RSSM13

Sr. No.	Biochemical test	Result
1	Gram character	-
2	Motility	+
3	Catalase	+
4	Oxidase	+
5	Lactose	-
6	Xylose	+
7	Maltose	+
8	Fructose	+
9	Dextrose	+
10	Galactose	-
11	Raffinose	-
12	Trehalose	-
13	Melibiose	-
14	Sucrose	+
15	L-Arabinose	+
16	Mannose	+
17	Inulin	+
18	Sodium gluconate	-
19	Glycerol	-
20	Salicin	+
21	Dulcitol	-
22	Inositol	-
23	Sorbitol	+
24	Mannitol	+
25	Adonitol	-
26	Arabitol	-
27	Erythiritol	-
28	Alpha-methyl-D-glucoside	-

Sr. No.	Biochemical test	Result
29	Rhamnose	-
30	Cellobiose	+
31	Melezitose	-
32	Alpha-Methyl-D-Mannoside	-
33	Xylitol	-
34	ONPG	-
35	Esculin	-
36	D-Arabinose	-
37	Citrate	-
38	Malonate	-
39	Sorbose	-
40	Indole	-
41	Methyl red	-
42	Voges Proskauers	-

By means of the different biochemical tests the isolate RSSM13 was tentatively identified to be *Burkholderia* sp.

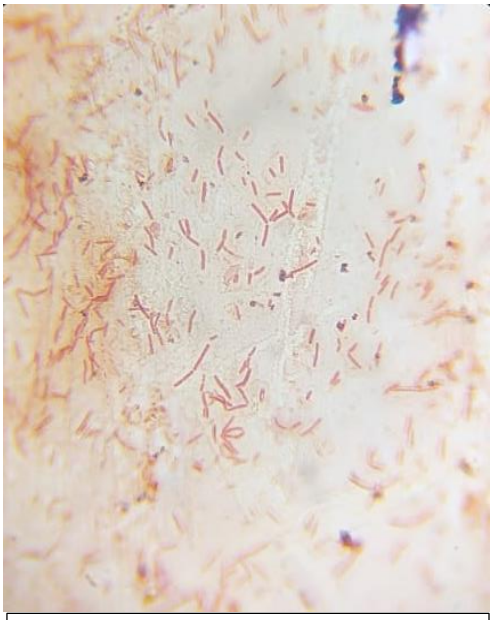

Fig 30. Gram negative-RSSM133

Fig 31. Motility test

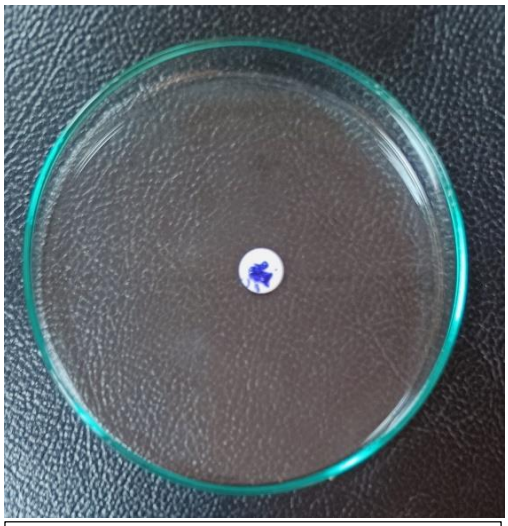

Fig 32. Oxidase test

Fig 33. Catalase test

Fig 34. IMViC test Kit

A- IMViC tests before addition of bacterial culture
B- IMViC tests 24 hours post addition of bacterial culture

Fig. 35 sugar utilization test kit

A- HiCarbo sugar utilization test before addition of bacterial culture
B- HiCarbo sugar utilization test 24 hour post addition of bacterial culture

4.10 Testing the *in-vivo* efficacy of selected biocontrol isolate-RSSM13

A wound of about 2 mm diameter was created on the leaf surface of the 4 treatments (positive control, negative control, test and untreated-uninfected control). Within 2 days browning appeared around positive control-chemical agent, negative control-no treatment and the test-rhizosphere associated bacterial isolate RSSM13. The lesion grew the largest in negative control which was without any treatment. The lesions developed on positive control and test were more or less equal in size.

Fig 36. Soil collected from Taleigao fields for pot trials

Fig 37. Pots in duplicates for the tests

Table 05-Lesion development over the days

	Disease progression over the days				
Treatments	**Day 02**	**Day 04**	**Day 06**	**Day 08**	**Day 10**
Positive control-Chemical treatment	4mm	4.6mm	4.6mm	4.6mm	4.6mm
Negative control-No treatment	7mm	8mm	8mm	9mm	9mm
Test- Bacterial isolate-RSSM13	3.4mm	4mm	4mm	4.4mm	4.4mm
Uninfected untreated control	2mm	2mm	2mm	2mm	2mm

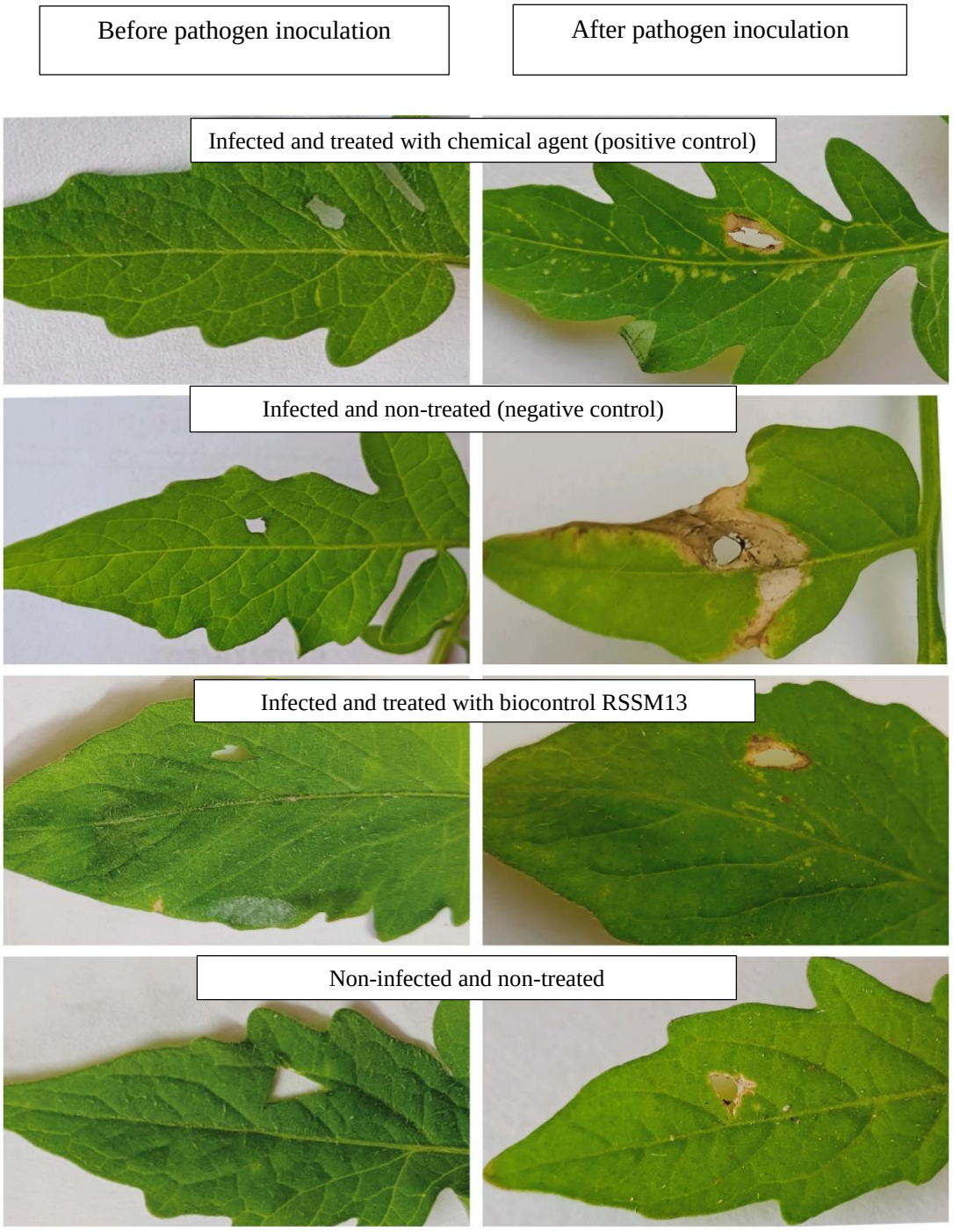

Fig 38. Disease development in tomato leaves with different treatment on day08

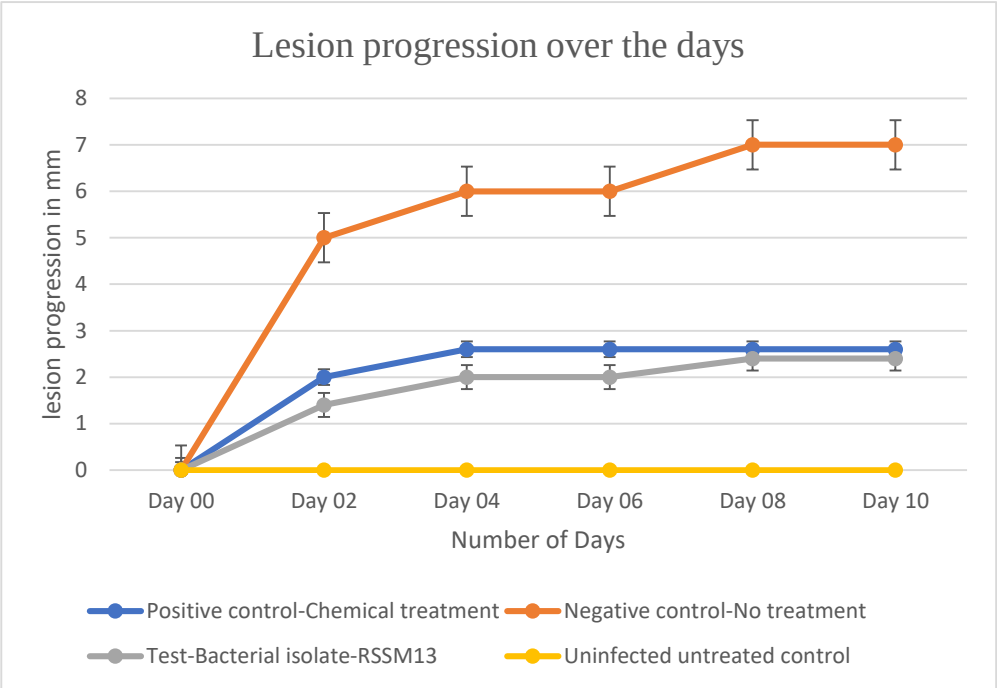

Fig 39. Graph showing disease progression

The trend depicted in the graph illustrates the progression of lesion diameter over time, specifically in response to different treatments. Initially, on day 0 no lesion progression was observed across any of the treatments. However, by day 2, a notable increase in lesion size was recorded, with negative control exhibiting the highest progression (5mm), followed by the positive control (2mm), and the test bacterial isolate RSSM13 (1.4mm).

Subsequently, on day 4, the lesion continued to expand, reaching 6mm in the negative control, 2.6mm in the positive control, and 2mm in the test isolate RSSM13. Interestingly, from day 4 to day 6, the lesion size remained constant across all treatments. However, by day 8, there was a resurgence in lesion progression observed in both the negative control (7mm) and test isolate (2.4mm).

By day 10, the lesion progression remained consistent with day 8 across all treatments. It's worth noting that no development or progression of lesions was observed in the uninfected

untreated control group but the infected leaves withered overtime and dried. Overall, the trend indicates a general increasing pattern in lesions diameter over time with variations in progression rates observed between different treatments.

The graph clearly depicts that the selected biocontrol agent RSSM13 is as effective as chemical agent in pot trials. The biocontrol agent also shows certain plant promotion properties. Hence the RSSM13 can be seen as a multi-faceted integrated pest management solution. This study carried out led to conclusion that RSSM13 bacteria isolated from the rhizospheric soil of healthy tomato plants showed maximum radial growth inhibition (by-50.6%) against phytopathogen *Myrothecium* sp. *in-vitro*. Similar study by Tenorio-Salgado et al.,2013 documented that *Burkholderia tropics* in particular strains MTo293 and TTe203 exhibited highest radial mycelial growth inhibition of pathogenic fungi *Colletotrichum gloeosporioides, Fusarium culmorum, Fusarium oxysporum and Sclerotium rolffs.* Also studies study carried out by Sunsaramoorthy and Balabaskar,2013 reveal that a fungi *Trichoderma harzaianum* (ANR-1) isolate effectively inhibited radial mycelial growth of pathogen (by 53%) *fusarium oxysporum* causing fusarium wilt. Under greenhouse conditions *Trichoderma harzaianum* showed significant disease reduction compared to control and also had plant stimulatory effects.

Conclusion

The ultimate isolate chosen in this investigation, denoted as RSSM13, has exhibited significant potential for mitigating disease both in controlled *in-vitro* settings and in pot trials specifically targeting Myrothecium sp., causing myrothecium leaf spot. RSSM13 not only showcased effectiveness in disease reduction but also displayed characteristics conducive to promoting plant growth, hinting at its capacity to foster healthier plant development. The RSSM13 isolate exhibited the synthesis of beneficial substances like chitinase and siderophore, akin to the findings of Narendra Babu et al. in 2015. Their study identified five plant growth-promoting rhizobacteria (PGPRs) from various genera, isolated from the rhizosphere of healthy tomato plants. These PGPRs demonstrated the ability to prevent early blight infection caused by *Alternaria solani*. Additionally, RSSM13 demonstrated the synthesis of lipase, ammonia, protease, and other compounds known for their proficiency in combating pathogens and fortifying plants against diseases. Consequently, based on the outcomes of our investigation, it is reasonable to propose RSSM13 as a promising candidate for employment as a microbial biocontrol agent in real-world agricultural settings, particularly in combatting foliar diseases affecting tomato crops. Moreover, the culture RSSM13 warrants further exploration to assess its efficacy against a spectrum of microbial pathogens affecting various agricultural crops, potentially expanding its utility and impact in agricultural practices.

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APPENDIX

Chemicals and Reagents used

Reagents Used	Source
Lactophenol Cotton Blue	HIMEDIA Laboratories pvt.
Congo red Dye	HIMEDIA Laboratories pvt.
Hydrogen Peroxide	HIMEDIA Laboratories pvt.
Sodium Hydroxide	HIMEDIA Laboratories pvt.
Hydrochloric acid	HIMEDIA Laboratories pvt.
Grams Iodine	HIMEDIA Laboratories pvt.
Grams Crystal violet	HIMEDIA Laboratories pvt.
Gram decolourizer	HIMEDIA Laboratories pvt.
Safranin	HIMEDIA Laboratories pvt.
Ethanol	HIMEDIA Laboratories pvt.
Oxidase disc	HIMEDIA Laboratories pvt

Media Used for isolation

Potato Dextrose Agar media – HIMEDIA

Ingredients	gms/l
Potato infusion	200
Dextrose (Glucose)	20
Agar	15
Final pH (at 25^{o}C)	5.6$\pm$0.2

Nutrient Agar- HIMEDIA

Ingredients	gms/l
Peptone	5
HM peptone	1.5
Yeast extract	1.5
NaCl	5
Agar	15
Final pH(at 25^{o}C)	7.4$\pm$0.2

Pikovskaya's Agar-HIMDEIA

Ingredients	gms/l
Yeast extract	0.5
Dextrose	10
Calcium phospahte	5
Ammonium sulphate	0.5
Potassium chloride	0.2
Magnesium sulphate	0.1
Manganese sulphate	0.0001
Ferrous sulphate	0.0001
Agar	15

SIM medium- HIMEDIA

Ingredients	gms/l
Peptone	30
HM Peptone B	3
Peptonized iron	0.2
Sodium thiosulphate	0.025
Agar	3
Final pH	7.3±0.2