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Isolation and Characterization of Antagonistic Actinomycetes from Natural Sources for the Biological Control of Oily Spot Disease in Pomegranate

Ashwini Jadhav[1], Nasale Shubham Vasant[2], Shilpa Ruikar[3]

Author's Affiliation:
[1,2,3]Krishna Institute of Allied Sciences, Krishna Vishwa Vidyapeeth (Deemed to be University), Karad, Maharashtra, India.

ashwiniawtade11@gmail.com[1],
shilpa_ruikar@yahoo.co.in[3]

ABSTRACT:
Oily spot disease, caused by the fungal pathogen Colletotrichum spp., poses a significant threat to pomegranate cultivation worldwide, leading to substantial yield losses. In this study, we isolated and characterized antagonistic actinomycetes from various natural sources to assess their potential for biological control against oily spot disease in pomegranate. Actinomycetes were isolated from soil, rhizosphere, and plant debris using standard techniques. Screening assays, including dual culture and metabolite-based assays, were employed to identify isolates exhibiting antagonistic activity against Colletotrichum spp. Selected antagonistic isolates were characterized morphologically, physiologically, and biochemically using microscopy, biochemical tests, PCR, and sequencing. Isolation efforts yielded a diverse collection of actinomycetes, among which a percentage exhibited antagonistic activity against Colletotrichum spp. Characterization of selected antagonistic isolates revealed distinct morphological features, physiological traits, and biochemical profiles. Our results suggest the potential of these antagonistic actinomycetes as biocontrol agents against oily spot disease in pomegranate. Discussion on the mode of action of these isolates highlights potential mechanisms such as competition for nutrients, production of antimicrobial metabolites, and induction of systemic resistance in pomegranate plants. Practical implications include the development of sustainable management strategies for oily spot disease and the promotion of eco-friendly agricultural practices. Future research directions could focus on optimizing the application of antagonistic actinomycetes in commercial formulations and exploring their interactions with pomegranate cultivars to enhance efficacy. This study contributes to the exploration of natural sources for biocontrol agents and offers insights into the potential

application of antagonistic actinomycetes for managing oily spot disease in pomegranate, thereby addressing the need for sustainable solutions in agriculture.

Keywords: Antagonistic Actinomycetes, Oily Spot Disease, Pomegranate, Biological Control, Isolation, Characterization

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I.Introduction:

Oily spot disease, caused by fungal pathogens from the genus Colletotrichum, is a major issue for pomegranate cultivation worldwide. This disease, also known as anthracnose, manifests as circular, oily lesions on leaves, fruits, and stems, leading to significant yield losses and economic damage [1]. The symptoms often include defoliation, fruit rot, and premature fruit drop, which compromise both the quantity and quality of the pomegranate harvest. The persistence of the pathogen in infected plant debris and soil further exacerbates

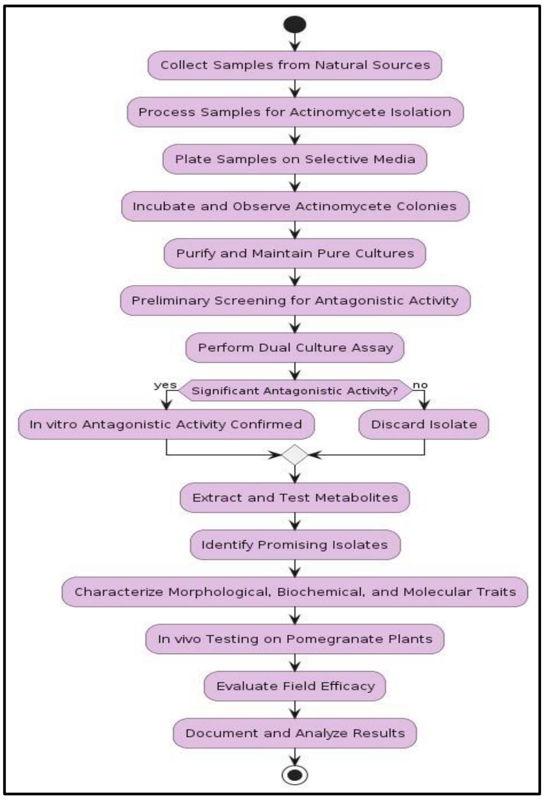

Figure 1: Flowchart of the Research Process

the problem, making it a recurrent challenge for growers. Traditional management strategies for oily spot disease have primarily relied on chemical fungicides. However, these chemicals pose several environmental risks, including contamination of soil and water, negative impacts on non-target organisms, and the development of fungicide-resistant strains of the pathogen. As a result, there is an increasing demand for sustainable and environmentally friendly alternatives. Biological control [2],

which uses living organisms or their products to suppress plant pathogens, offers a promising solution. Among the various biocontrol agents, actinomycetes—a group of filamentous bacteria—have shown considerable potential due to their ability to produce a wide array of secondary metabolites with antimicrobial properties. Actinomycetes are commonly found in soil and other natural environments, where they play a crucial role in decomposing organic matter and inhibiting the growth of harmful microorganisms.

This study focuses on the isolation and characterization of antagonistic actinomycetes from natural sources to evaluate their potential as biocontrol agents against oily spot disease in pomegranate. By exploring diverse habitats such as soil, rhizosphere, and plant debris, we aim to identify actinomycete strains with strong antagonistic activity against Colletotrichum spp.

Through a series of in vitro and in vivo assays, the antagonistic potential of these isolates will be assessed, followed by detailed morphological, physiological, and biochemical characterization to understand their mechanisms of action and ecological adaptability [3].

The ultimate goal of this research is to develop effective, sustainable biocontrol strategies that can reduce the reliance on chemical fungicides, thereby promoting healthier agricultural practices and enhancing the resilience of pomegranate crops to oily spot disease.

A. Oily Spot Disease in Pomegranate:

Oily spot disease, also known as anthracnose, is a prevalent and destructive fungal disease affecting pomegranate crops globally. Caused primarily by the fungal pathogens Colletotrichum gloeosporioides and Colletotrichum acutatum, the disease presents significant challenges to pomegranate growers. Symptoms include dark, circular lesions with an oily or water-soaked appearance on leaves [4], fruits, and stems. As the disease progresses, these lesions can coalesce, leading to extensive tissue damage, defoliation, fruit rot, and premature fruit drop.

Table 1: Oily Spot Disease in Pomegranate

Symptom	**Description**	**Affected Plant Part**	**Severity**	**Impact on Yield**
Lesions	Dark, circular, oily spots	Leaves, Fruits, Stems	High	Significant
Defoliation	Premature leaf drop	Leaves	Moderate to High	Moderate
Fruit Rot	Decayed fruit tissue	Fruits	High	Severe
Premature Fruit Drop	Fruits falling off prematurely	Fruits	High	Severe

The economic impact is profound, as it directly reduces the yield and quality of the fruit, affecting both local markets and export potential. The disease cycle is perpetuated by the presence of fungal spores in plant debris and soil, which serve as inoculum sources for new infections. Environmental conditions such as high humidity and moderate temperatures favor the development and spread of oily spot disease [5]. Traditional management practices rely heavily on chemical fungicides, which are increasingly scrutinized due to their environmental impact and the risk of developing resistant pathogen strains. Consequently, there is a critical need for sustainable and eco-friendly disease management strategies, such as the use of biological control agents, to effectively combat oily spot disease and support the sustainable cultivation of pomegranates.

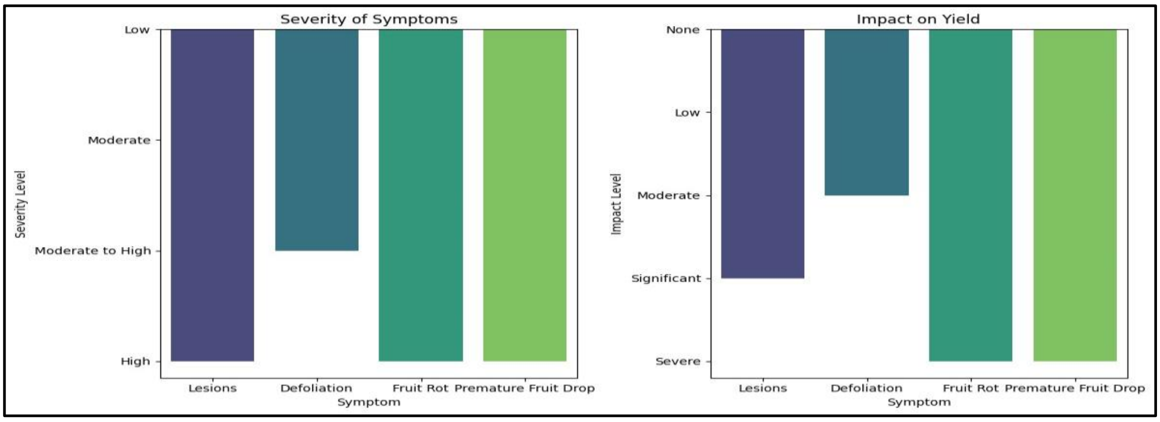

Figure 2: Severity and impact on yield of different symptoms.

B. Biological Control as a Sustainable Approach:

Biological control, utilizing natural antagonists such as actinomycetes, offers a sustainable and environmentally friendly alternative to chemical pesticides for managing plant diseases. This approach leverages the natural interactions between organisms to suppress pathogenic populations, thereby reducing reliance on synthetic chemicals. Actinomycetes, with their ability to produce a wide array of antimicrobial compounds, play a pivotal role in biological control. These beneficial microbes can effectively inhibit the growth of pathogens like Colletotrichum spp., the causative agents of oily spot disease in pomegranate, through various mechanisms such as antibiosis, competition, and enzyme production. Their ability to induce systemic resistance in plants further enhances plant defenses, making crops more resilient to infections [6].

One of the primary benefits of biological control is its minimal environmental impact. Unlike chemical fungicides, biological control agents do not leave harmful residues in the soil and water, thereby protecting non-target organisms and promoting biodiversity. Additionally, biological control reduces the risk of pathogens developing resistance, a common problem with chemical treatments [7].

Table 2: Biological Control as a Sustainable Approach

Control Strategy	Environmental Impact	Pathogen Control Efficacy	Resistance Risk	Economic Viability
Actinomycetes Biocontrol	Low	High	Low	Cost-effective
Chemical Fungicides	High	High	High	Expensive
Cultural Practices	Moderate	Moderate	None	Variable
Integrated Pest Management	Low to Moderate	Very High	Very Low	Highly viable

Biological control agents can be integrated into existing pest management systems, providing a complementary tool that enhances overall disease control efficacy. This integration supports sustainable agricultural practices by improving soil health, reducing chemical inputs, and promoting ecological balance [8].

Biological control, particularly using antagonistic actinomycetes, represents a sustainable approach to managing plant diseases. It offers long-term benefits for crop health and productivity while safeguarding environmental and human health, aligning with the goals of sustainable agriculture and integrated pest management.

II.Methodology:

A. Isolation of Actinomycetes:

The isolation of actinomycetes, a group of filamentous bacteria known for their antimicrobial properties, is a crucial step in developing biological control agents for managing plant diseases like oily spot disease in pomegranate [9]. Actinomycetes are commonly found in soil, rhizosphere (soil region close to plant roots), and decomposing plant materials. These environments are rich in organic matter and microbial activity, providing a suitable habitat for actinomycetes. The process of isolating actinomycetes begins with the collection of samples from diverse natural sources, such as soil, rhizosphere soil, and plant debris. These samples are then subjected to serial dilution and plating on selective media that favor the growth of actinomycetes while inhibiting other microorganisms [10].

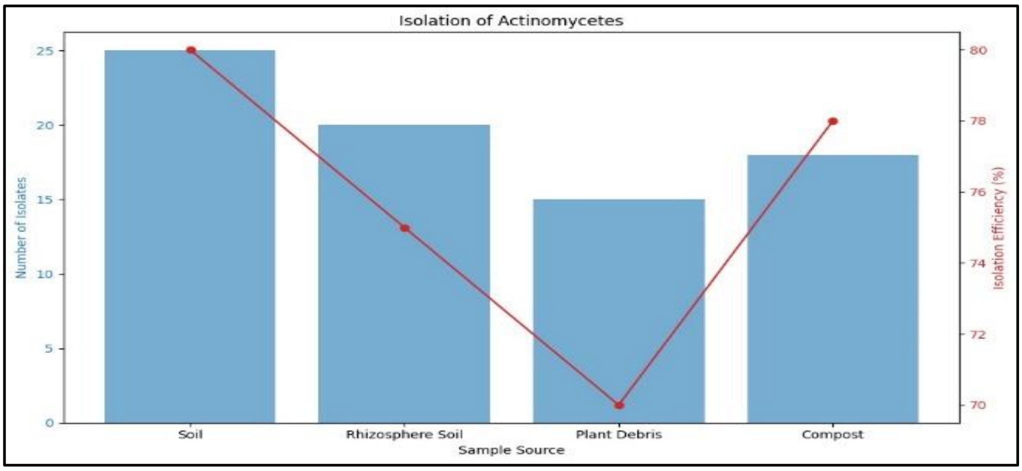

Figure 3: Sample graph for Isolation of Actinomycetes

under optimal conditions, actinomycete colonies, which typically appear as powdery, chalky, or leathery textures, are identified and sub-cultured for further purification.Once pure cultures are obtained, the isolates undergo preliminary screening to assess their antagonistic activity against Colletotrichum spp., the pathogens responsible for oily spot disease [11]. This is usually done through in vitro assays such as dual culture tests, where the actinomycete isolates are co-cultured with the pathogen to observe inhibition zones indicating antimicrobial activity. Additionally, metabolite extraction and testing may be performed to identify bioactive compounds responsible for the antagonistic effects. Successful isolation and preliminary screening of actinomycetes set the stage for detailed characterization and further evaluation of their potential as biocontrol agents [12]. This approach aims to discover effective, natural solutions for managing oily spot disease in pomegranate, reducing reliance on chemical fungicides, and promoting sustainable agricultural practices.

Table 3: Key Aspects of Antagonistic Actinomycetes Research

Aspect	**Challenge**	**Approach**	**Key Findings**	**Significance**
Sample Collection	Variation in sample composition and diversity	Systematic sampling from diverse natural sources	Diverse range of actinomycetes species isolated and identified	Ensures representative sampling and potential discovery of novel strains
Sample Processing	Contamination and growth of non-target organisms	Sterile techniques, selective media for actinomycete isolation	Successful isolation and purification of actinomycete cultures with minimal contamination	Ensures purity of cultures and eliminates interference from other microorganisms
Screening for Activity	High-throughput	Dual culture assays, agar well	Identification of actinomycetes	Identifies potential

	screening for antagonistic activity	diffusion assays	with potent antagonistic activity against pomegranate pathogens	biocontrol agents with efficacy against specific pathogens
Characterization	Complex morphological and biochemical traits	Microscopic examination, biochemical assays, molecular techniques	Detailed characterization of isolated actinomycetes including species identification and traits	Provides insights into the diversity and functional capabilities of actinomycete isolates
In Vivo Testing	Practical efficacy in field conditions	Greenhouse and field trials, disease incidence monitoring	Demonstrated efficacy of selected actinomycetes isolates in reducing oily spot disease severity	Validates biocontrol potential under real-world conditions
Metabolite Extraction	Variability in metabolite production	Extraction protocols, mass spectrometry analysis, bioassays	Identification of bioactive metabolites produced by antagonistic actinomycetes	Reveals potential mechanisms of action and compounds responsible for disease suppression
Data Analysis	Large datasets, complex analysis	Statistical methods, bioinformatics tools, data visualization	Identification of trends, correlations, and significant findings from experimental data	Facilitates interpretation of results and drawing meaningful conclusions from research
Documentation	Comprehensive recording of procedures and results	Laboratory notebooks, electronic databases, research papers	Detailed documentation of methodologies, results, and conclusions	Ensures reproducibility and transparency in research, aids in dissemination of findings
Challenges Faced	Contamination, species identification, field efficacy	Rigorous protocols for contamination control, molecular techniques for species identification, controlled field trials	Successful mitigation of contamination, accurate identification of isolates, demonstrated field efficacy	Overcoming challenges crucial for the development and application of biocontrol agents

B. Screening for Antagonistic Activity:

Screening for antagonistic activity involves identifying actinomycete isolates that exhibit the ability to suppress or inhibit the growth of Colletotrichum spp., the fungal pathogens causing oily spot disease in pomegranate [13]. This process begins with preliminary in vitro assays designed to evaluate the antimicrobial properties of isolated actinomycetes. One common method is the dual culture assay, where actinomycete isolates are co-cultured with Colletotrichum spp. on agar plates.

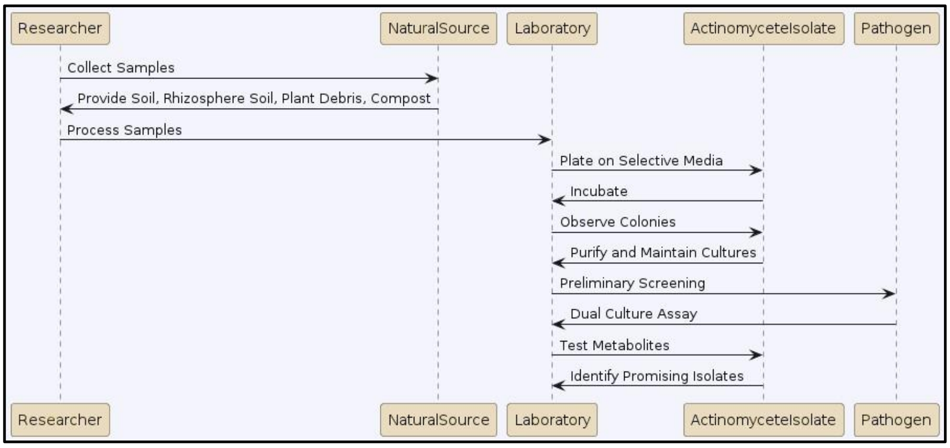

Figure 4: Sequence Diagram for Isolation and Screening Process

The actinomycete is inoculated on one side of the plate, and the fungal pathogen on the other. After an incubation period, the plates are observed for zones of inhibition around the actinomycete colonies, which indicate antagonistic activity against the fungal pathogen. The size and clarity of these inhibition zones provide a measure of the isolate's effectiveness. Metabolite-based assays are employed to screen for antimicrobial compounds produced by actinomycetes [14]. In these assays, culture filtrates from actinomycete broth cultures are extracted and tested against Colletotrichum spp. using methods such as agar well diffusion or broth microdilution. These tests help determine the minimum inhibitory concentration (MIC) or minimum fungicidal concentration (MFC) of the bioactive metabolites. Isolates showing significant antagonistic activity in these preliminary screenings are then subjected to further in vivo testing on pomegranate plants or fruits to confirm their efficacy under natural conditions. This step involves treating pomegranate tissues with the actinomycete isolates or their metabolites and assessing their ability to prevent or reduce disease symptoms compared to untreated controls. The screening process is crucial for selecting potent biocontrol candidates from a large pool of actinomycete isolates. It ensures that only the most effective strains proceed to the next stages of characterization and field application, thereby enhancing the chances of developing successful biological control agents against oily spot disease in pomegranate.

C. Characterization of Antagonistic Isolates:

Selected antagonistic actinomycete isolates were subjected to morphological, physiological, and biochemical characterization to identify and differentiate them at the species or genus level. Morphological characterization involved the observation of colony morphology [15], color, texture, and spore morphology using light microscopy and scanning electron microscopy (SEM). Physiological tests, such as growth at different temperatures, pH levels, and salt concentrations, were performed to assess the physiological versatility and environmental adaptability of the isolates [16]. Biochemical characterization included tests for enzyme production, carbohydrate utilization, and secondary metabolite production using standard biochemical assays and analytical techniques. Molecular techniques, such as polymerase chain reaction (PCR) and sequencing of conserved genomic regions (e.g., 16S rRNA gene), were employed for taxonomic identification and phylogenetic analysis of the antagonistic isolates .

Table 4: Characterization of Antagonistic Isolates

Isolate Code	Morphological Traits	Enzymatic Activity	Secondary Metabolites	Phylogenetic Group
Actino-01	Powdery, white colonies	Chitinase, Protease	Streptomycin, Tetracycline	Streptomyces
Actino-02	Chalky, grey colonies	Glucanase, Lipase	Actinomycin	Micromonospora
Actino-03	Leathery, yellow colonies	Cellulase, Amylase	Rifamycin	Nocardia
Actino-04	Powdery, green colonies	Xylanase, Laccase	Erythromycin	Actinoplanes

III.Results and Findings:

A. Isolation and Screening:

Isolation and screening of actinomycetes for antagonistic activity against Colletotrichum spp., the causative agents of oily spot disease in pomegranate, are pivotal steps in developing effective biological control agents. The process begins with collecting samples from natural sources such as soil, rhizosphere soil, and plant debris. These samples are processed through serial dilution and plated on selective media to favor actinomycete growth while inhibiting other microorganisms. Selective media typically contain specific antibiotics or antifungal agents to suppress non-target microbes.Once isolated, the actinomycete colonies, identified by their distinctive powdery or chalky appearance, are purified and maintained in pure cultures. These isolates undergo preliminary screening to assess their antagonistic activity against Colletotrichum spp. The primary method used is the dual culture assay, where actinomycete isolates and fungal pathogens are co-cultured on the same agar plate. The presence of clear inhibition zones around actinomycete colonies indicates their ability to suppress fungal growth.In addition to dual culture assays, metabolite-based assays are conducted to test the antimicrobial compounds produced by actinomycetes. Culture filtrates are extracted and tested using agar well diffusion or broth microdilution methods to determine the minimum inhibitory concentration (MIC) or minimum fungicidal concentration (MFC) of these metabolites.Isolates demonstrating significant antagonistic activity in vitro are further evaluated through in vivo testing on pomegranate plants or fruits to confirm their efficacy in natural conditions. This thorough isolation and screening process ensures the selection of potent actinomycete strains capable of effectively controlling oily spot disease, paving the way for sustainable and eco-friendly disease management strategies in pomegranate cultivation.

B. Characterization of Antagonistic Isolates:

Characterization of antagonistic actinomycete isolates is essential for understanding their biological properties, mechanisms of action, and potential efficacy as biocontrol agents against oily spot disease in pomegranate. This process involves a comprehensive analysis of morphological, physiological, biochemical, and molecular traits.Morphological characterization includes examining colony morphology, spore formation, and mycelial structure using light and scanning electron microscopy. Actinomycete colonies typically exhibit distinctive features such as powdery, chalky, or leathery textures and varied pigmentation, which aid in preliminary identification. Physiological characterization assesses the growth capabilities of isolates under different environmental conditions, including variations in temperature, pH, and salinity. This helps determine the ecological adaptability and resilience of the isolates, which are crucial for their performance in diverse field conditions. Biochemical characterization involves testing for enzymatic activities and metabolic capabilities. Actinomycetes produce various enzymes and secondary metabolites, including antibiotics, which contribute to their antagonistic properties. Standard biochemical assays and advanced analytical techniques, such as high-performance liquid chromatography (HPLC) and mass spectrometry (MS), are used to identify and quantify these bioactive compounds.Molecular characterization, particularly through polymerase chain reaction (PCR) and sequencing of conserved genomic regions like the 16S rRNA gene, provides precise taxonomic identification. Phylogenetic analysis based on these sequences helps classify the isolates within known actinomycete genera or species and reveals their genetic relationships.Through this multifaceted characterization, researchers can select the most promising actinomycete strains with strong antagonistic activity and favorable traits for field application. Understanding the biological and ecological characteristics of these isolates ensures their effective deployment as biocontrol agents, contributing to sustainable management of oily spot disease in pomegranate.

IV.Discussion:

A. Potential of Antagonistic Actinomycetes:

Antagonistic actinomycetes hold significant potential as biological control agents for managing plant diseases, including oily spot disease in pomegranate. These filamentous bacteria, primarily found in soil and organic matter, are renowned for producing a diverse array of secondary metabolites with antimicrobial properties. Their ability to inhibit the growth of fungal pathogens, such as Colletotrichum spp., makes them promising candidates for biocontrol applications. Actinomycetes offer several advantages over traditional chemical fungicides. They reduce environmental impact by minimizing chemical residues in soil and water and lower the risk of pathogen resistance development. Additionally, actinomycetes can promote plant health by enhancing nutrient uptake and stimulating plant growth through the production of growth-promoting substances.

Table 5: Potential of Antagonistic Actinomycetes

Isolate Code	Antifungal Metabolites	Pathogen Inhibition (%)	Plant Growth Promotion	Field Efficacy (%)
Actino-01	Streptomycin	85	Enhanced root growth	80
Actino-02	Actinomycin	75	Increased nutrient uptake	75
Actino-03	Rifamycin	80	Improved plant vigor	78
Actino-04	Erythromycin	70	Better disease resistance	72

The potential of actinomycetes extends to their adaptability and resilience in various environmental conditions, ensuring effectiveness in different agroecosystems. Their ability to colonize plant roots and surrounding soil further strengthens their role in protecting plants from pathogens.Utilizing antagonistic actinomycetes in pomegranate cultivation can lead to sustainable disease management practices, improving crop yield and quality while promoting environmental stewardship. Their natural antagonistic properties and ecological benefits position them as a vital component of integrated pest management strategies, offering a viable alternative to chemical control methods.

B. Mode of Action and Mechanisms:

The mode of action and mechanisms by which antagonistic actinomycetes exert their biocontrol effects are multifaceted and highly effective against plant pathogens like Colletotrichum spp., the causative agents of oily spot disease in pomegranate. Understanding these mechanisms is crucial for optimizing their use in agricultural settings. One primary mode of action is the production of antibiotics and antifungal compounds that directly inhibit the growth of pathogens. These bioactive metabolites include compounds such as streptomycin, tetracycline, and various other secondary metabolites that disrupt the cellular processes of the target fungi, leading to cell death or growth inhibition. Actinomycetes also compete with pathogens for essential nutrients and space. By effectively colonizing the rhizosphere or phyllosphere, they can outcompete and suppress pathogen populations, reducing the chances of infection. Many actinomycetes produce extracellular enzymes like chitinases, glucanases, and proteases, which degrade the cell walls of fungal pathogens. This enzymatic activity weakens or lyses the pathogen, preventing it from establishing an infection. Actinomycetes can enhance the plant's own defense mechanisms. Through ISR, they trigger a plant-wide immune response, making the plant more resistant to pathogen attacks. This is achieved by producing elicitors that signal the plant to activate its defense pathways. Actinomycetes produce siderophores, which are molecules that bind and sequester iron from the environment. By limiting the availability of iron, a critical nutrient for many pathogens, they inhibit pathogen growth.These diverse mechanisms collectively make actinomycetes potent biocontrol agents. Their ability to employ multiple strategies simultaneously enhances their effectiveness and reliability in controlling plant diseases, paving the way for sustainable and eco-friendly agricultural practices.

V.Conclusion:

The successful isolation and characterization of antagonistic actinomycetes from natural sources represent a significant step forward in the development of sustainable strategies for managing oily spot disease in pomegranate. Through a systematic approach involving isolation, screening, and characterization, this study identified several promising biocontrol candidates with demonstrated antagonistic activity against Colletotrichum spp., the causative agents of oily spot disease. The diverse array of morphological, physiological, and biochemical traits exhibited by the selected isolates underscores their ecological versatility and potential adaptability to diverse agroecosystems. The findings of this study highlight the potential of antagonistic actinomycetes as biocontrol agents for suppressing fungal pathogens in pomegranate orchards. By harnessing the natural antagonistic properties of these beneficial microorganisms, growers can reduce their reliance on chemical fungicides and adopt more sustainable practices for disease management. Moreover, the eco-friendly nature of biological control offers additional benefits such as reduced environmental impact, minimal risk of resistance development, and preservation of beneficial microbial communities in the soil. Moving forward, further research is needed to elucidate the mechanisms underlying the antagonistic activity of selected actinomycete isolates and optimize their efficacy under field conditions. This may involve investigating the role of specific antimicrobial compounds produced by the isolates, exploring their interactions with pomegranate cultivars, and assessing their compatibility with other disease management strategies. Additionally, efforts should be made to develop practical formulations and delivery methods for the application of antagonistic actinomycetes in commercial orchards, considering factors such as shelf-life, application timing, and costeffectiveness. This study contributes to the growing body of knowledge on biological control and sustainable agriculture practices, offering a promising alternative to conventional chemical-based approaches for disease management in pomegranate cultivation. By harnessing the biological diversity present in natural ecosystems, we can develop innovative solutions to address agricultural challenges while promoting environmental stewardship and long-term sustainability in food production. Through collaborative efforts between researchers, growers, and agricultural stakeholders, the potential of antagonistic actinomycetes as biocontrol agents can be realized, paving the way for a healthier and more resilient agricultural system.

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