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Assessment of the Effectiveness of Biocontrol Agents Against Bacterial Pathogens Affecting *Punica granatum*

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ABSTRACT

Pomegranate (*Punica granatum*) stands as a highly esteemed table fruit globally, cherished for its commercial, medicinal, and nutritional significance. India emerges as a key contributor to the production of pomegranates, with Maharashtra leading the nation's production. However, pomegranate growers in India face substantial economic threats due to the absence of effective control measures against diseases affecting the crop. Biological control emerges as a promising approach for managing plant diseases, necessitating the exploration of safe biocontrol agents.

In light of this context, we conducted a study involving the collection of samples from pomegranate orchards in Maharashtra, focusing on leaves, fruits, buds, and branches. Our objective was to assess the bacterial population and isolate Trichoderma species from these samples to evaluate their potential as biocontrol agents for pomegranate crops. Pomegranate crops are susceptible to various bacterial and fungal pathogens, leading to a reduction in fruit market value. Traditional methods of disease control involving bactericides and fungicides pose risks to public health and are not always cost-effective.

Hence, there is a growing need for safe and efficient biocontrol agents, with Trichoderma species emerging as promising candidates in this regard. In our study, we assessed the biocontrol potential of different Trichoderma species, namely *Trichoderma viride RSS1, Trichoderma reesei RSS2, and Trichoderma stercorarium RSS3*, against bacterial pathogens affecting pomegranate crops, including *Xanthomonas axonopodis MSR1, Xanthomonas campestris MSR2, and Xanthomonas vesicatoria MSR3*. Our findings demonstrate the efficacy of these Trichoderma species as potential biocontrol agents against pomegranate bacterial pathogens, paving the way for sustainable disease management strategies in pomegranate cultivation.

Keywords : Pomegranate, *Trichoderma species*, Plants extract, *Xanthomonas species*, Antibacterial activity.

INTRODUCTION

Controlling plant diseases is crucial for maintaining the quality and quantity of food, feed, and fiber worldwide. Various methods are employed to prevent, mitigate, or manage plant diseases. While chemical fertilizers and pesticides have historically played a significant role in enhancing crop productivity and quality, concerns over environmental pollution and public perception have led to a shift in attitudes towards pesticide use in agriculture. Today, stringent regulations and political pressure aim to reduce the use of hazardous chemicals in farming.

In response to these challenges, researchers are exploring alternative pest and disease management strategies, including biological control. Biological control, also known as biocontrol, involves using living organisms, or their products, to suppress plant diseases. This approach often utilizes antagonistic microbes to combat plant pathogens, and it may also involve the use of host-specific pathogens for weed control. Importantly, biocontrol agents must be safe for both humans and the environment.

In this study, we investigated the antibacterial properties of aqueous extracts from Azadirachta indica, Pongamia pinnata, and Ricinus communis plants against Xanthomonas vesicatoria MSR3 using the agar well diffusion assay. Additionally, we evaluated the biocontrol potential of different Trichoderma species, including *Trichoderma viride RSS1, Trichoderma reesei RSS2,* and *Trichoderma stercorarium RSS3,* against bacterial pathogens affecting pomegranates, such as *Xanthomonas axonopodis MSR1, Xanthomonas campestris MSR2,* and *Xanthomonas vesicatoria MSR3,* using the disc diffusion assay.

MATERIALS AND METHODS

Preliminary identification and isolation of the pathogen from affected parts of Pomegranate plant

Initial identification and isolation of the pathogen from affected parts of the pomegranate plant involved the implementation of an ooze test, following the methodology outlined by Sharma *et al*., (2010), which relies on microscopic observations to confirm the presence of the pathogen in infected leaves. Leaves exhibiting typical symptoms of oily spots (bacterial blight) were collected from the field.

In this test, the infected leaves underwent a series of procedures: they were initially washed with sterile distilled water, followed by sterilization with 0.1% mercuric chloride ($HgCl_2$) for 10 minutes, and then repeatedly washed with sterile distilled water before being blotted dry. Subsequently, fresh young lesions containing leaves were selected, and small bits of infected tissue were excised from affected parts such as leaves and pericarp of the fruits using a sterile scalpel. These tissue bits were then placed in a drop of sterile saline on a glass slide and observed under a high-power objective of a compound microscope. The microscopic examination aimed to detect the presence of bacterial ooze, characterized by the release of bacterial cells from the cut tissue into the surrounding water, as described by Schaad (1992).

Furthermore, bacterial suspensions prepared from infected tissues obtained from affected leaves, twigs, and fruits were utilized to isolate distinct bacterial colonies on suitable growth media. Singh *et al*., (2015) emphasized that disease diagnosis relies on visual symptoms observed on plant parts in conjunction with the ooze test.

Isolation of bacteria exudes out from ooze

The infected leaf was washed and subsequently crushed in sterile distilled water. The resulting extract was then streak-inoculated onto the surface of sterile Glucose Yeast Extract Calcium Carbonate Agar (GYECC Agar), following the method described by Yenjerappa (2009). The inoculated plate was then incubated at 30^0C for a period of 3 days. Based on the literature, colonies that appeared well isolated, gummy, mucous, and exhibited a yellow coloration were chosen for further investigation (Yenjerappa, 2009).

Isolation of *Trichoderma species*

Soil samples were gathered from various ecological habitats within Latur district, India, with the aim of isolating Trichoderma spp. These soil specimens were transported to the laboratory and stored at 4^0C until required. Subsequently, four-fold serial dilutions of each soil sample were prepared using sterilized distilled water. A volume of 0.5ml of these diluted samples was then spread onto the surface of Potato Dextrose Agar (PDA), following the protocol outlined by Elad *et al*., (1982). The plates were then placed in an incubator set at 28 ± 2^0C for duration of 96 hours. Morphologically distinct colonies observed on the agar plates were selected and purified on fresh Potato Dextrose Agar (PDA). The purified and promising isolates were further preserved at 4^0C for subsequent use throughout the study.

RESULTS AND DISCUSSIONS

Trichoderma, a filamentous fungus found in soil, is extensively employed as a biocontrol agent due to its ability to parasitize various plant pathogenic fungi. Among the Trichoderma species, *T. harzianum, T. viride, and T. asperellum* are commonly cited as effective biocontrol agents against plant pathogens (Harman *et al*., 2004, 2011; Kumar *et al*., 2012; Cuervo-Parra *et al*., 2011).

Table 1: *In Vitro* Evaluation of Antibacterial Potential of Biocontrol Agents against Bacterial Pathogens of Pomegranate Diseases

Bacterial Pathogens	*Trichoderma viride RSS1*	*Trichoderma reesei RSS2*	*Trichoderma stercorarium RSS3*
	Zone of Growth Inhibition (mm)		
Xanthomonas axonopodis MSR1	42	40	39
Xanthomonas campestris MSR2	40	37	35
Xanthomonas vesicatoria MSR3	38	33	30

***In Vitro* Evaluation of Biocontrol Agents against Bacterial Pathogens**

Trichoderma species are commonly found inhabitants of the rhizosphere and play a significant role in controlling numerous soil-borne plant diseases caused by fungi. The potential of various Trichoderma species as biological agents against plant diseases has been recognized since the 1930s. Therefore, three species of Trichoderma were selected for their antifungal activity against the targeted pathogens as follows.

1) *Trichoderma viride RSS1*

2) *Trichoderma reesei RSS2*

3) *Trichoderma stercorarium RSS3*.

Three biocontrol agents, namely *Trichoderma viride RSS1*, *Trichoderma reesei RSS2*, and *Trichoderma stercorarium RSS3*, were evaluated against three bacterial pathogens, namely *Xanthomonas axonopodis MSR1*, *Xanthomonas campestris MSR2*, and *Xanthomonas vesicatoria MSR3*, respectively isolated from diseased pomegranate leaves, stems, and fruits. The zone of growth inhibition was measured and calculated, with the results presented in **Table 1**.

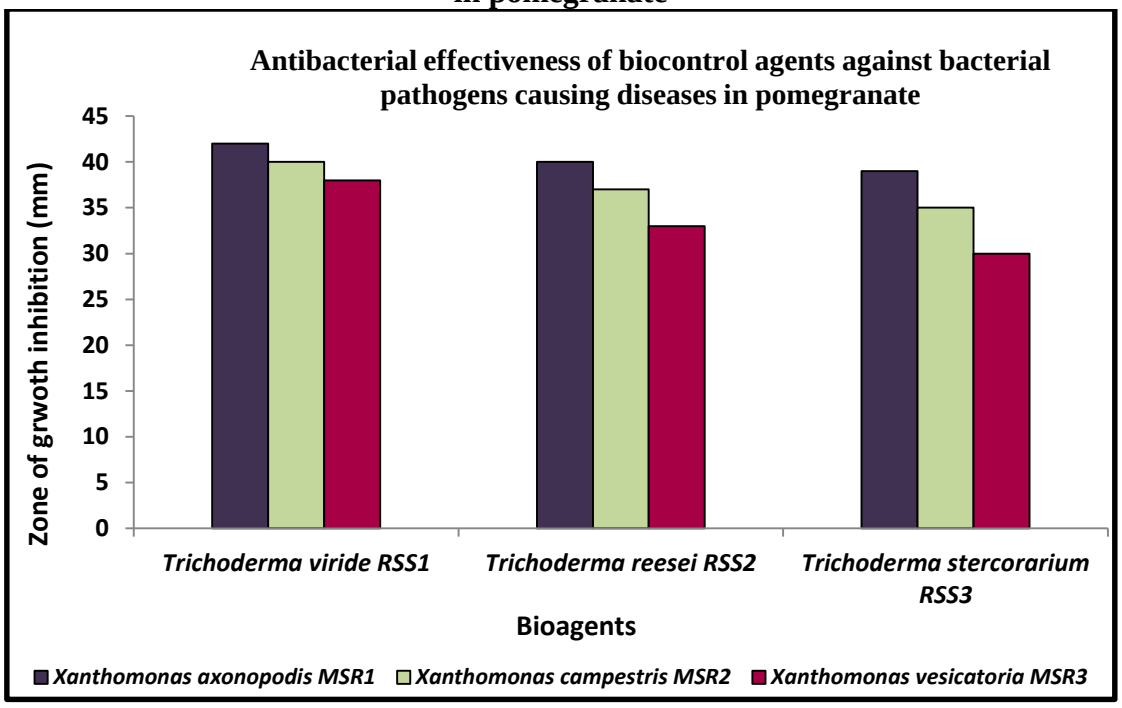
Among the three bioagents tested (as shown in **Table 1**), *Trichoderma viride RSS1* demonstrated the highest level of inhibition against the tested pathogens.

Evaluation of Efficacy of Biocontrol agents against Bacterial Pathogen

In the current context, where the use of chemical pesticides in plant disease management faces various constraints, biological control has emerged as a highly valued and increasingly recognized alternative. This approach is gaining prominence due to its eco-friendly nature and cost-effectiveness. Within the realm of biological control, various antagonistic organisms have been identified, each employing a range of mechanisms such as competition, antibiosis, siderophore production, and lysis to combat pathogens.

In the present investigation, Trichoderma viride exhibited the highest level of inhibition against the three bacterial pathogens, namely *Xanthomonas axonopodis MSR1*, *Xanthomonas campestris MSR2*, and *Xanthomonas vesicatoria MSR3*. This observation underscores the potential effectiveness of Trichoderma viride as a biocontrol agent in mitigating plant diseases caused by bacterial pathogens.

Figure 1: Antibacterial effectiveness of biocontrol agents against bacterial pathogens causing diseases in pomegranate



The antibacterial potential of three biocontrol agents, namely *Trichoderma viride RSS1*, *Trichoderma reesei RSS2*, and *Trichoderma stercorarium RSS3*, against bacterial pathogens, specifically *Xanthomonas axonopodis MSR1*, *Xanthomonas campestris MSR2*, and *Xanthomonas vesicatoria MSR3* responsible for pomegranate disease, was assessed. The results are illustrated in **Figure 1**.

Analysis of **Figure 1** reveals that all three biocontrol agents, namely *Trichoderma viride RSS1*, *Trichoderma reesei RSS2*, and *Trichoderma stercorarium RSS3*, exhibited significant zones of inhibition. Specifically, *against Xanthomonas axonopodis MSR1*, the maximum zone of inhibition recorded was 42mm,

40mm, and 39mm, respectively. Similarly, against *Xanthomonas campestris MSR2*, the maximum zones of inhibition were 40mm, 37mm, and 35mm, respectively, for the three bioagents. Likewise, against *Xanthomonas vesicatoria MSR3*, the maximum zones of inhibition were 38mm, 33mm, and 30mm, respectively, for the same bioagents. These findings highlight the effectiveness of Trichoderma species as potential biocontrol agents against bacterial pathogens associated with pomegranate disease.

Our findings align closely with those reported by Nurbailis *et al*., (2019), who demonstrated the efficacy of *Trichoderma viride, Trichoderma harzianum, and Trichoderma PP3* in reducing the growth of *Xanthomonas axonopodis pv*., the causal agent of leaf blight disease in red onion.

Abhishek Gupta *et al*., (2018) documented that the secondary metabolites produced by *Trichoderma viride*, notably cellulase enzyme, exhibit potent antibacterial activity against *Xanthomonas citri*, a plant pathogenic bacterium responsible for causing disease in lemon plants. This suggests the potential of cellulase enzyme as a biocontrol agent against *Xanthomonas citri* infection in lemon plants.

Our findings contradict those reported by Bhure *et al*., (2019), who observed different antagonistic activities among three bioagents. According to their study, *Trichoderma harzianum* exhibited the maximum inhibition zone (22.86mm), followed by *Pseudomonas fluorescens* (17.20mm) and *Bacillus subtilis* (15.00mm).

Molecular Identification of Biocontrol Agents

The molecular identification of the biocontrol agents was conducted through 18s rRNA sequencing. The full-length 18s rRNA region was amplified using universal primers. Subsequently, the PCR-generated sequences were analyzed using the BLASTn algorithm available at the National Center for Biotechnology Information (NCBI) website (www.ncbi.nlm.nih.gov/BLAST) to identify closely related sequences.

Related sequences for fungal isolates were retrieved from the NCBI database and aligned using CLUSTAL X2 and DAMBE multiple sequence alignment tool. The phylogenetic evolutionary relationships were then inferred using the Kimura-2-parameter and Neighbor Joining Method analysis.

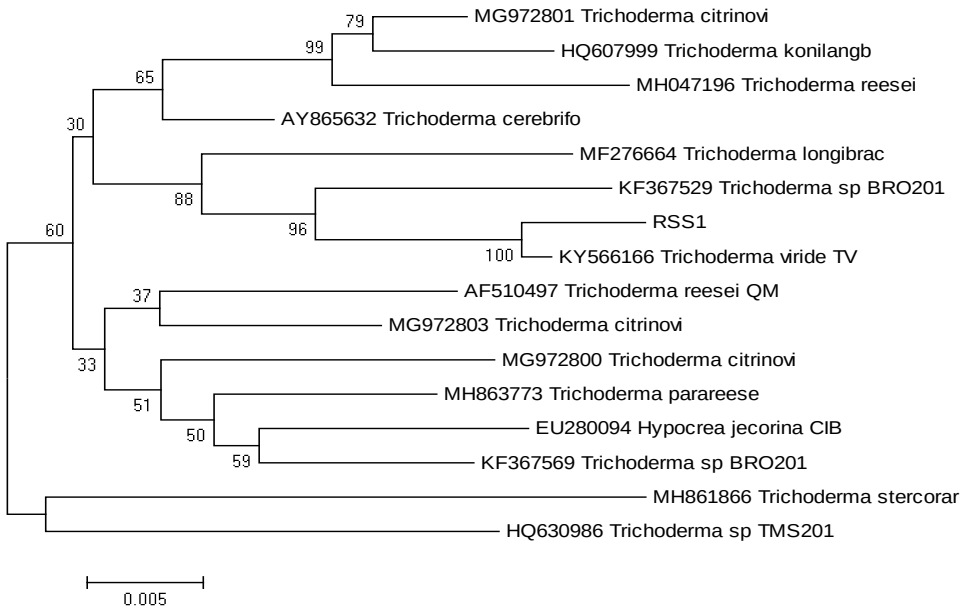


Figure 2: Phylogenetic placement of RSS1 based on 18S rRNA analysis

The phylogenetic tree of *Trichoderma viride RSS1* was constructed based on the analysis of its 18s rRNA gene sequence. Bootstrap support values, expressed as percentages, are depicted at the nodes of the tree, representing the confidence levels derived from neighbor-joining analyses with 1,000 replicates. Additionally, the scale bar (0.005) on the tree indicates the genetic distance between sequences.

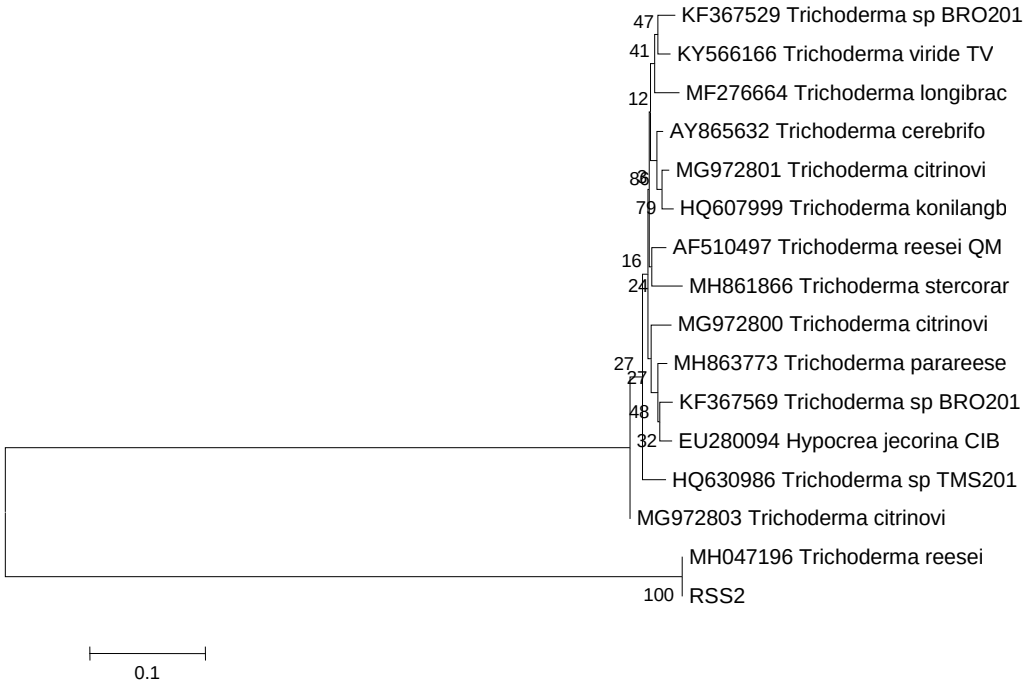


Figure 3: Phylogenetic placement of RSS2 based on 18S rRNA analysis

A phylogenetic tree was constructed for *Trichoderma reesei RSS2* based on the analysis of its 18s rRNA gene sequence. The percent values displayed at the nodes represent the levels of bootstrap support derived from neighbor-joining analyses conducted with 1,000 replicates. Additionally, the scale bar (0.1) on the tree illustrates the genetic distance between sequences.

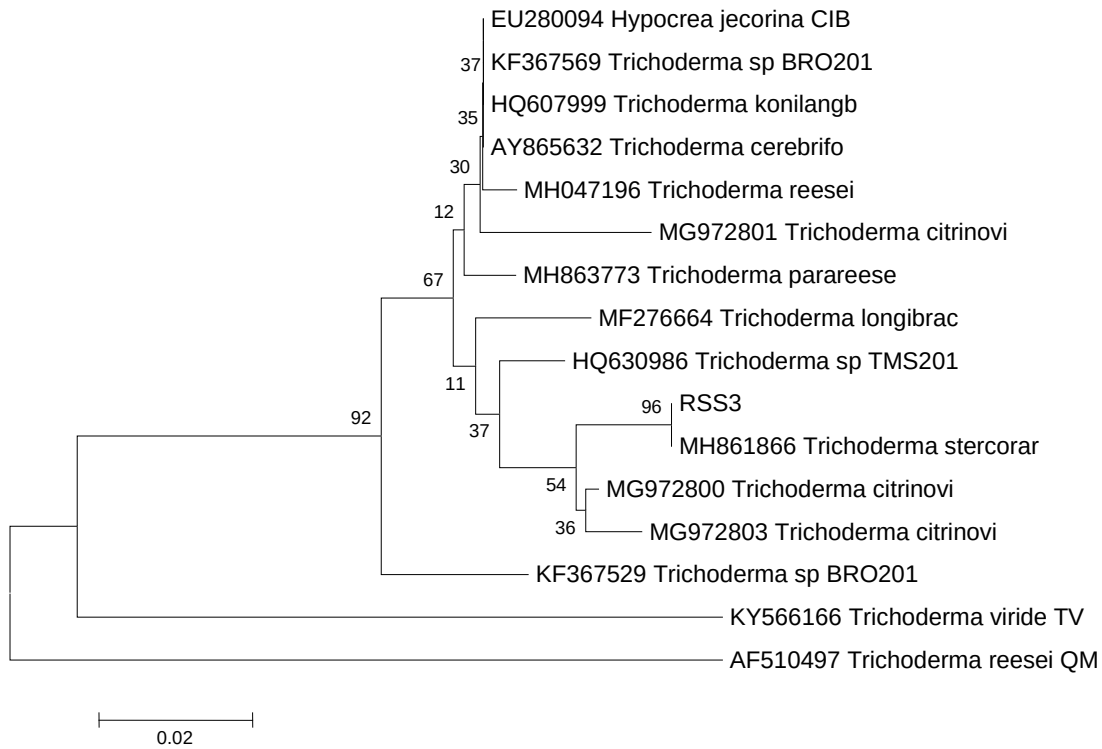


Figure 4: Phylogenetic placement of RSS3 based on 18S rRNA analysis

A phylogenetic tree was generated for *Trichoderma stercorarium RSS3* through the analysis of its 18s rRNA gene sequence. Bootstrap support percentages at the nodes reflect the confidence levels obtained from neighbor-joining analyses conducted with 1,000 replicates. Furthermore, the scale bar (0.02) on the tree denotes the genetic distance between sequences.

Shreeshail *et al*., (2015) conducted an in vitro study evaluating the efficacy of seven botanical extracts, including Neem leaf extract, Garlic bulb extract, Onion bulb extract, Datura leaf extract, Ocimum leaf extract, Eucalyptus leaf extract, and Rhizome extract, along with four bioagents, namely *Bacillus subtilis*, *Pseudomonas fluorescens*, *Trichoderma viride*, and *Trichoderma harzianum*, against the causal agent of pomegranate wilt, *Ceratocystis fimbriata*.

Their findings indicated that among the four bioagents tested, *Trichoderma harzianum* and *Trichoderma viride* exhibited 100% inhibition of *Ceratocystis fimbriata*. *Pseudomonas fluorescens* also demonstrated notable inhibition (42.33%) within 4 days, completely suppressing perithecium production and inhibiting pathogen growth.

Our findings contradict those reported by Shreeshail *et al*., (2015). In our study, among the different bioagents tested, *Trichoderma harzianum* exhibited the highest level of inhibition against the causal agent of pomegranate wilt, *Ceratocystis fimbriata*, achieving a 100% inhibition rate. Interestingly, *Trichoderma viride* also showed comparable efficacy, achieving a similar 100% inhibition rate against the test fungus.

Antibiotic-mediated suppression is a primary mechanism observed in biocontrol, where biocontrol agents produce one or more antimicrobial compounds that hinder the growth of various plant pathogens. For instance, *Bacillus cereus* strain is recognized for its production of zwittermycin (Silo-Suh *et al*., 1994) and kanosamine (Milner *et al*., 1996; Silo-Suh *et al*., 1998). The capacity to generate multiple antibiotics enables biocontrol agents to inhibit a broad spectrum of plant pathogens, thereby enhancing the effectiveness of biological control.

Pseudomonas putida WCS358r, a genetically engineered strain, produces phenazine and 2,4-Diacetylphloroglucinol (DAPG), which have been shown to enhance disease suppression in field conditions (Glandorf *et al*., 2001).

CONCLUSIONS

In these interactions, pathogens are countered by other organisms, indicating the involvement of multiple mechanisms in biological control. These mechanisms include hyperparasitism or predation, antibiotic production, lytic enzymes, unregulated waste products, and induction of host resistance.

Hyperparasitism or predation involves the direct attack of specific biocontrol agents on pathogens, leading to their elimination. Additionally, biocontrol agents produce one or more antimicrobial compounds that hinder the growth of various plant pathogens. The ability of biocontrol agents to produce multiple antibiotics enables them to target a broad spectrum of plant pathogens, thereby enhancing the efficacy of biological control.

The effectiveness of biological control has been demonstrated through the biocontrol potential of different Trichoderma species, including *Trichoderma viride RSS1*, *Trichoderma reesei RSS2*, and

Trichoderma stercorarium RSS3, against bacterial pathogens affecting pomegranates such as *Xanthomonas axonopodis MSR1*, *Xanthomonas campestris MSR2*, and *Xanthomonas vesicatoria MSR3*.

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