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Harnessing Microbial Consortium for Effective Control of *Xanthomonas axonopodis pv. punicae*-Induced Bacterial Blight of Pomegranate

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ABSTRACT

The occurrence of blight disease in pomegranate cultivation, caused by *Xanthomonas axonopodis (pv. Punicae)*, has posed a significant challenge. This disease manifests in symptoms on leaves, fruits and stems, ultimately leading to a substantial reduction in yield, often by as much as 70-75%. We are actively seeking an environmentally friendly and cost-effective organic approach to manage blight disease in pomegranates that is both beneficial to farmers and safe for consumers. In this study, the pathogen responsible for the disease was extracted from a diseased pomegranate fruit and leaves obtained from Hosadurga, Karnataka. The isolation process was carried out on nutrient agar media through the streak plating method. A literature review was conducted to identify potential microorganisms suitable for the creation of a consortium. A list of these organisms was obtained from various laboratories. Subsequently, each microorganism was evaluated for compatibility and based on the results the consortium was formulated. The compatible microorganisms included in the consortium are *Bacillus subtilis, Pseudomonas spp, Trichoderma spp* and *Penicillium spp.* The antagonistic activity of the isolated causative organism was tested with each organism in the consortium and with the prepared consortium. The study initially commenced with in vitro experiments and later progressed to greenhouse trials. To assess antagonistic activity, the formation of inhibition zones in millimeters was measured, resulting in the following values *Bacillus subtilis* (15.2±0.185 mm), *Pseudomonas spp*. (9.9±0.121 mm), *Trichoderma spp*. (16.5±0.306 mm) and *Penicillium spp*. (12.5±0.426 mm). The developed microbial consortium demonstrated the most promising result with an inhibition zone of 25±0.121 mm. Encouraged by these findings the consortium was selected for greenhouse studies. Upon the onset of disease in the plants the consortium was applied via spraying at five-day intervals for a duration of 30 days. Fruit yield results were compared with those from control plants that received no treatment. Ultimately the developed consortium exhibited a positive impact on fruit yield and healthy leaves were observed.

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Key Words

Bacterial blight, *bacillus subtilis*, microbial consortium, *pseudomonas spp, penicillium spp, trichoderma spp*

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INTRODUCTION

The pomegranate (*Punica granatum L*.), often referred to as the "Fruit of Paradise," holds ancient significance and is a major tropical and subtropical fruit crop. Originating in Iran, it has been cultivated extensively across the Mediterranean, including countries like Spain, Morocco and Egypt, as well as in regions such as Afghanistan, Arabia and Baluchistan. This fruit carries historical and mythical weight, appearing in various mythologies and stories. It is a round, leathery-rinded fruit that contains juicy arils and is prized for its unique flavor and potential health benefits due to its rich antioxidant content. Beyond its nutritional value the pomegranate's symbolism of abundance and fertility has cemented its cultural importance, making it a celebrated fruit across diverse societies. The pomegranate fruit is widely cherished for its delicious and revitalizing juice. The matured fruit's arils, or seed clusters, are enjoyed both fresh and in various processed forms, including juices, concentrates, syrups and jellies. Moreover, the seeds and fleshy portions of sour pomegranates are dried and sold as "Anardana," a sought-after condiment known for its tangy flavor and often used to enhance curries and dishes. This versatility in consumption underscores the fruit's culinary significance and role in enhancing the taste of a wide array of dishes.

The rapid expansion of pomegranate cultivation in India reflects its growing importance as a cash crop with significant economic potential. Pomegranate has gained recognition not only for its adaptability but also for its nutritional value and versatility in various culinary and industrial applications. The statistics from the 2013-14 agricultural year provide a snapshot of its prominence in India's agriculture sector. The global expansion of pomegranate cultivation represents a remarkable agricultural success story driven by the fruit's unique characteristics and economic potential. Pomegranates are renowned for their resilient nature, making them well-suited for cultivation in a wide range of environmental conditions. Their ability to thrive in arid tropical and subtropical regions has endeared them to farmers in regions where water resources are limited. In a world facing increasing water scarcity due to climate change the drought tolerance of pomegranate plants is a significant advantage, offering a reliable source of income for farmers even in challenging conditions.

Another key factor contributing to the global popularity of pomegranates is their ability to yield abundantly while also possessing exceptional storage capabilities. Pomegranates can be stored for extended periods without significant quality degradation, making them an attractive option for both domestic and export markets. This feature ensures a stable supply of pomegranates throughout the year, contributing to their consistent demand and economic viability. The economic significance of pomegranates is further bolstered by their favorable prices in various markets. Pomegranates are sought after for their unique taste, high nutritional value and versatility in culinary applications. Whether consumed fresh, as juice, or as processed products like jams and sauces, pomegranates have found their way into the hearts and palates of consumers worldwide. This broad appeal drives market demand and enhances the income potential for growers, positioning pomegranates as a valuable commodity in the global agricultural landscape.

However, this cultivation faces challenges, including the onslaught of pests and diseases. Bacterial blight, caused by *Xanthomonas axonopodis pv. Punicae* Vauterin *et al*. poses a significant threat. Since 2002, this disease has reached critical levels, severely affecting India's economy, particularly the export of quality fruits. By 2007, due to the disease's impact, pomegranate production in India had dropped by 60% following an outbreak that resulted in a 70-100% loss in Karnataka and Maharashtra during 2006 Raghavan, 2007. This underscores the need for effective disease management strategies to secure the pomegranate industry. Disease and Pest Management: Disease and pest management pose significant challenges to pomegranate cultivation. Blight disease, caused by bacteria like *Xanthomonas axonopodis pv. Punicae*, can lead to devastating crop losses if not effectively controlled. Pesticides have traditionally been used to combat these issues but there is a growing concern about the environmental and health impacts of chemical treatments. Therefore, pomegranate growers are increasingly exploring alternative approaches, such as biocontrol agents, organic farming methods and integrated pest management (IPM) strategies to mitigate the risks associated with disease and pests.

Organic pomegranate growers are actively seeking alternatives to synthetic pesticides to control diseases like bacterial blight while maintaining the integrity of organic farming practices. These alternatives encompass a range of strategies, including the use of plant-derived botanicals, beneficial microorganisms and bio-agents. Botanicals such as neem oil, garlic extracts and essential oils from aromatic herbs have demonstrated potential as organic disease-control agents. Likewise, beneficial microorganisms, such as certain species of bacteria and fungi, can play a pivotal role in suppressing pathogens and enhancing plant immunity. One of the most promising avenues for organic disease management in pomegranates is the use of microbial consortia. These consortia, composed of multiple microorganisms working synergistically, align perfectly with organic farming principles. They offer a holistic approach to disease control, combining

antagonistic activity against pathogens the stimulation of plant defense mechanisms and the enhancement of soil health. Additionally, microbial consortia do not leave harmful residues in the environment, making them well-suited for organic systems.

MATERIALS AND METHODS

Collection of sample: To study blight disease, we gathered fresh samples displaying symptoms from infected *Punica granatum* (pomegranate) variety Bhagwa from the Hosadurga region. We made sure to collect these samples from various locations within the orchard to ensure a comprehensive and representative selection. These samples were carefully placed in sterile plastic bags to maintain their integrity during transport and they were subsequently transported to the laboratory for in-depth analysis.

Surface sterilization: The collected plant samples undergo surface sterilization to remove external contaminants. They are first washed under running tap water to remove soil and debris. Then, they are immersed in a 70% ethanol solution for 1 minute and subsequently in a 0.1% $HgCl_2$ solution for 20 Seconds to eliminate microorganisms. Finally, the samples are rinsed three times with sterile distilled water to ensure they are free from contaminants before use in laboratory experiments or analyses.

Tissue homogenization: The next step in the process involves aseptically cutting the surface-sterilized plant tissues into small pieces and homogenizing them using a sterile mortar and pestle. This homogenization step is crucial for breaking down the plant material into a uniform consistency for further analysis or experimentation. To maintain sterility, the homogenization is conducted within a sterile laminar flow hood to prevent contamination from external airborne particles or microorganisms. This controlled environment ensures the integrity of the plant samples and the accuracy of subsequent procedure.

Isolation of pathogen: To isolate the bacteria, a loop ful of the resulting leachate, containing bacterial cells, was streaked aseptically onto Yeast Dextrose Calcium Carbonate Agar (YDCA) plates. These plates were then incubated at a specified temperature (30±10°C) for 48 hrs. Colonies that grew within this time frame were carefully selected and streaked again onto YDC agar plates. Discrete colonies were sub-cultured on YDC agar slants for further analysis. To maintain the cultures, they were periodically renewed through sub-culturing every two weeks on YDC agar slants. This comprehensive protocol ensured the successful isolation and cultivation of bacteria from the pomegranate plant samples displaying bacterial infection symptoms for subsequent research and study.

Purification and maintenance of bacterial culture: The suspected bacterial colonies were carefully selected using sterilized inoculation loops and then streaked onto the surface of Petri plates containing Yeast Extract Dextrose Calcium Carbonate Agar Schaad and Stall, 1988. These Petri plates were sterilized to ensure a controlled environment for bacterial growth. The inoculated plates were subsequently incubated at a temperature of 30°C for a duration of 72 hrs. During the incubation period, observations were made to identify the development of well-separated, typical, bright yellow and mucoid colonies. These pure colonies, which displayed the desired characteristics, were then further streaked onto agar slants containing a nutrient agar medium. These newly streaked cultures were again incubated at 30°C for 72 hrs. To preserve the cultures for future studies, they were stored in a refrigerator at 5°C, effectively serving as a stock culture. For long-term storage the bacterium was added to sterile distilled water and kept at a temperature of -4°C, ensuring the viability of the bacterial cultures over an extended period. This meticulous process allowed for the isolation, characterization and preservation of the bacterial strains for subsequent research and analysis.

Identification and characterization of the pathogen: The identification of the bacterium causing pomegranate bacterial blight involved a thorough examination of its morphological, biochemical, cultural and molecular traits using standard microbiological techniques.

Morphological characters: The morphological traits of the pathogen, including cell shape, gram reaction, pigmentation, presence of capsules and spore staining, were investigated following established protocols outlined in references by Anon 1957, Bradbury 1970 and Schaad 1992.

Biochemical characterization of pomegranate bacterial blight causing pathogen: Various biochemical tests, including starch hydrolysis, gelatin liquefaction, hydrogensulfide production, KOH solubility, utilization of carbon sources, pectolytic enzyme activity acid production from organic compounds, milk proteolysis, acid production from carbohydrates, urease production and more, were conducted following the procedures detailed in Schaads 1992 methods.

Isolation of genomic dna from pathogen: Isolating genomic DNA from *Xanthomonas axonopodis pv.* punicae, a bacterial pathogen that causes disease in pomegranate plants, typically involves a series of steps that include cell lysis, DNA extraction and purification.

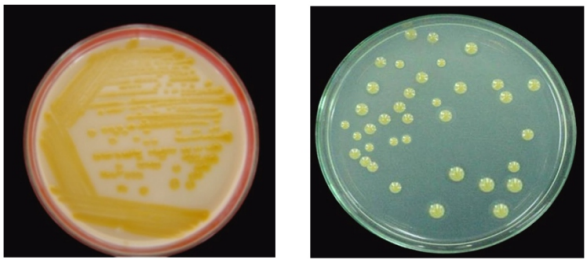

Fig. 1: Xanthomonas axonopodis pv punicae pure culture

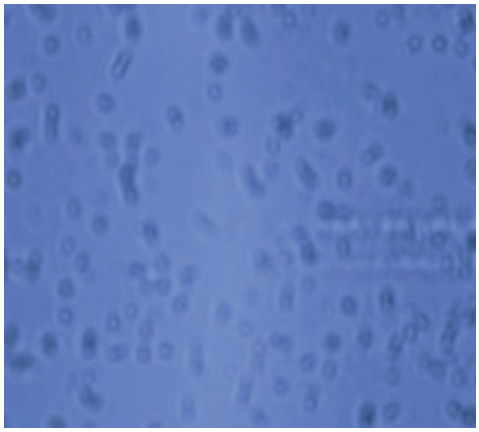

Fig. 2: Microscopic view at 100 X

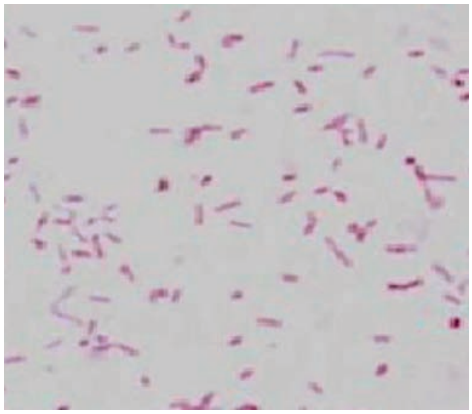

Fig. 3: Gram Staining

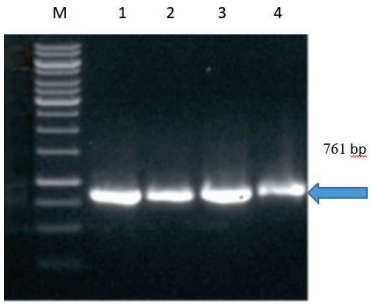

Fig. 4: PCR Amplification with universal Primer

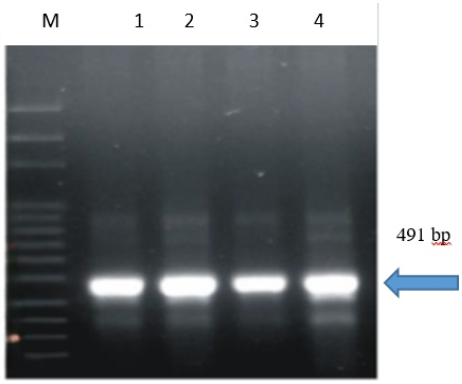

Fig. 5: PCR Amplification with gyrB Primer

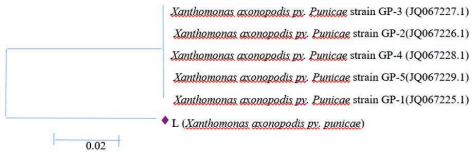

Fig. 6: Phylogenetic tree showing the relatedness to the original strain and the isolated strains

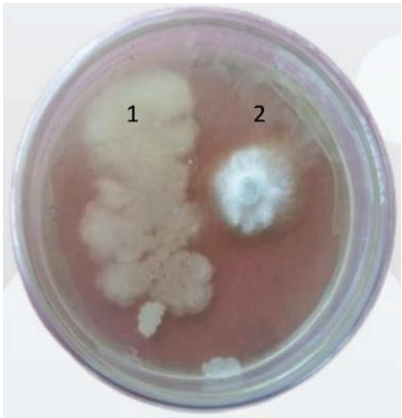

Fig. 7: Compatibility test of 1(*B.subtilis*) with 2 (*Trichoderma spp*)

The isolated genomic DNA from Xap is now ready for downstream applications such as PCR, DNA sequencing, or restriction enzyme digestion. The DNA was stored at-20°C or-80°C for long-term preservation and further analysis.

Amplification and sequencing of 16s RRNA gene: Amplifying and sequencing the 16S rRNA gene of *Xanthomonas axonopodis pv. punicae* is a common method for bacterial identification and phylogenetic

Fig. 8: Compatibility test of 1(*Pseudomonas*) with 2 (*Trichoderma spp*)

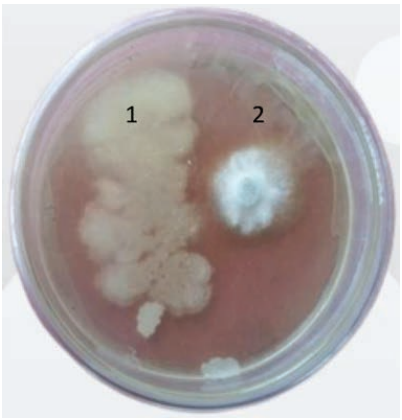

Fig. 11: Compatibility test of 1(B.subtilis) with 2 (Penicilllium spp)

Fig. 9: Compatibility test of 1(*B.subtilis*) with 2 (*Pseudomonas spp*)

Fig. 12: Compatibility test of 1 (B.subtilis) with 2 (Penicillium spp)

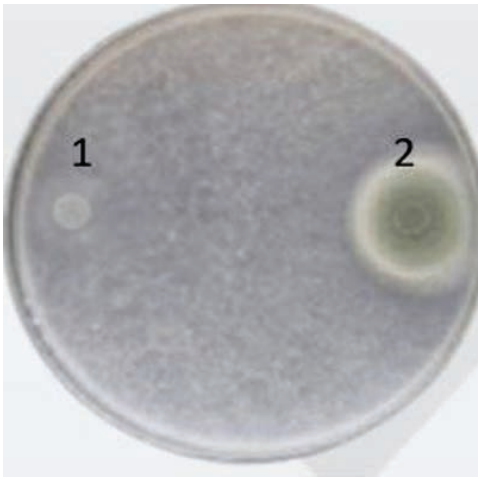

Fig. 10: Compatibility test of 1(Penicillium) with 2 (Trichoderma spp)

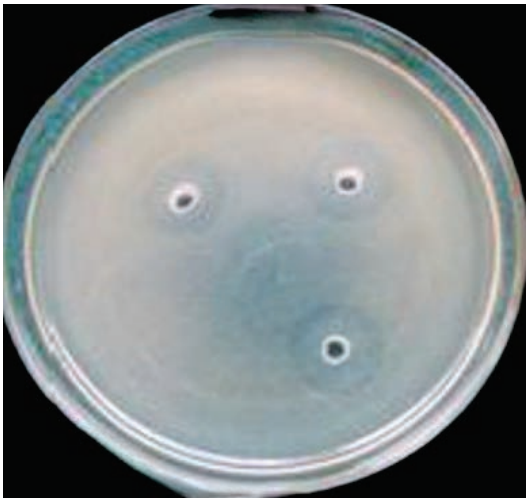

Fig. 13: Antagonistic activity of Bacillus subtilis against Xap

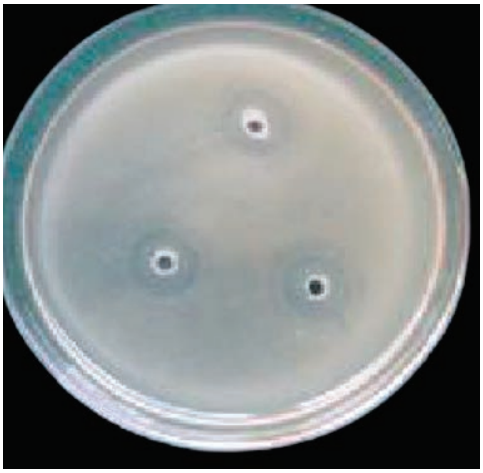

Fig. 14: Antagonistic activity of Bacillus subtilis against Xap

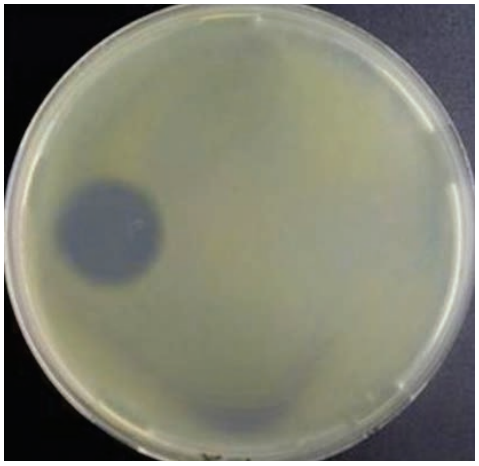

Fig. 15: Antagonistic activity of Pseudomonas spp against Xap

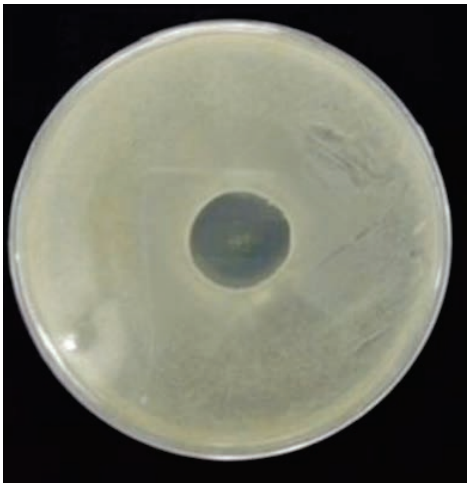

Fig. 16: Antagonistic activity of Trichoderma spp against Xap

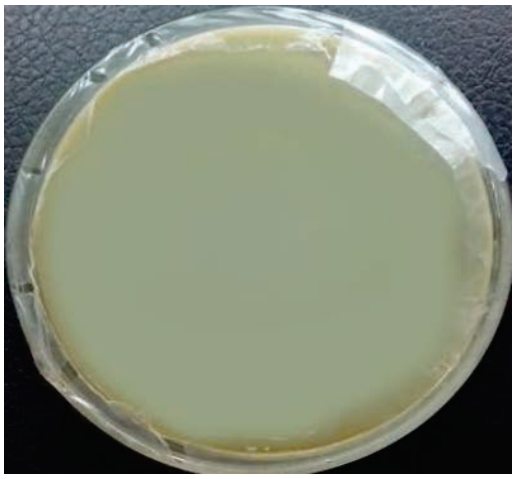

Fig. 17: Antagonistic activity of Penicillium spp against Xap

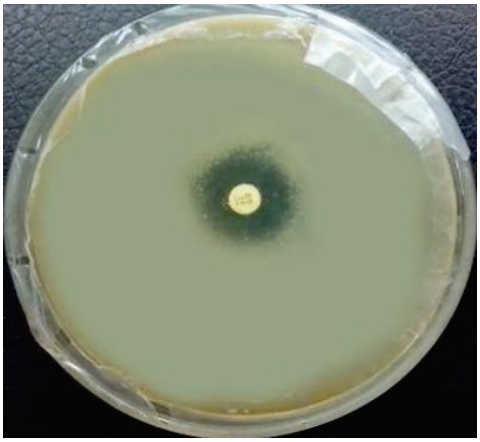

Fig .18a: Control

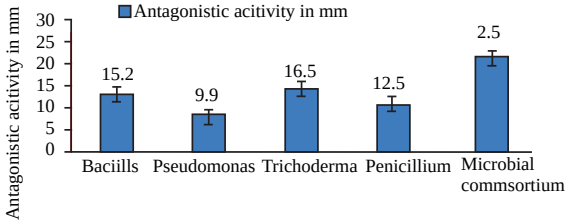

Fig. 18b: Antagonistic activity of formulated Microbial consortium asainst Xap

analysis. This protocol outlines the key steps for amplifying the 16S rRNA gene using PCR, verifying the analysis. This protocol outlines the key steps for amplifying the 16S rRNA gene using PCR, verifying the presence of the amplified products through agarose gel electrophoresis and visualizing them with ethidium bromide staining under UV light. The choice of primer,

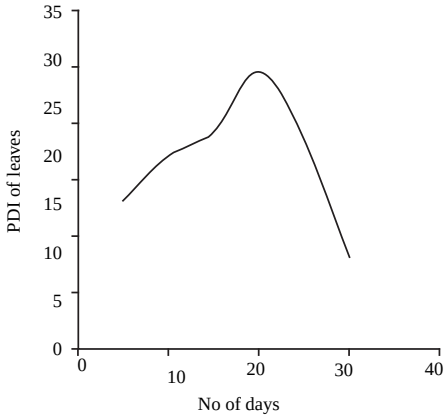

Fig. 19: Comparison of antagonistic activity of individual organisms in the consortium v/s consortium

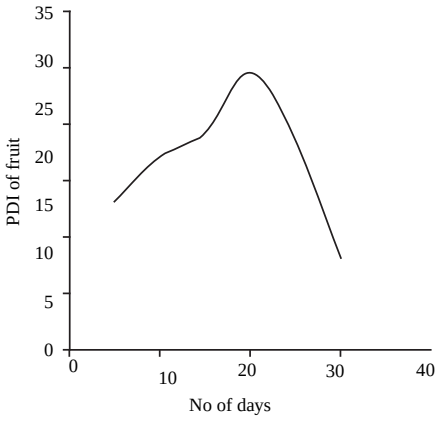

Fig. 20: Plot showing decrease in the PDI of Leaves after application of Consortium

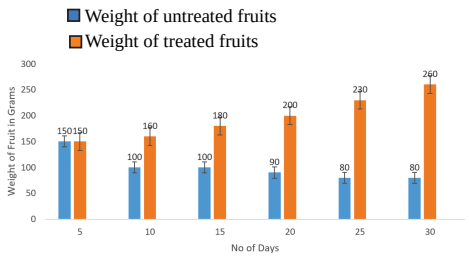

Fig. 21: Plot showing decrease in the PDI of Leaves after application of Consortium

annealing temperature and other conditions are critical for successful amplification of the target gene. In this case the annealing temperature is 46°C and the primers used are designed for the 16S rRNA gene region of *Xanthomonas axonopodis pv. punicae*. The unpurified PCR products to Amnion Biotech Pvt. Ltd. for sequencing of the 16S rRNA gene. After receiving the sequencing data, BioEdit software was used to reverse complement the sequences and then aligned them using Clustal X software. This alignment process helps analyze the genetic relationships between individual bacteria and can be used for various purposes such as phylogenetic analysis and identifying conserved regions in the gene. In the next stage of analysis, bioinformatics tools and databases were used for bacterial identification and phylogenetic analysis. The first step involved using the BLAST (Basic Local Alignment Search Tool) program to compare the aligned DNA sequences of the 16S rRNA gene with the extensive Gen Bank database hosted by the National Center for Biotechnology Information (NCBI). This process enables the program to identify the most closely related DNA sequences in the database, thereby aiding in the identification of bacterial species. Additionally, reference sequences were downloaded from the EMBL database through the NCBI website to support the comparisons.

Notably, stricter identity criteria of 97-99% were applied when identifying bacteria, ensuring a high degree of similarity with their closest relatives in the NCBI database. Furthermore, to visualize the evolutionary relationships between these bacterial strains, a phylogenetic tree was constructed using the Mega 6 software based on the data obtained from the NCBI query. This comprehensive approach allowed us to effectively determine the species and phylogenetic relationships of the bacteria present in the infected pomegranate.

Preparation of microbial consortia: Screening for Compatibility: Compatibility testing of bacterial and fungal isolates is a pivotal stage in microbiology and biotechnology, essential for selecting and optimizing microbial combinations for diverse applications. The process involves meticulously selecting isolates of interest and cultivating them under controlled conditions on agar plates or in liquid media. The primary goal is to determine if these isolates can coexist harmoniously without impeding each other's growth or antagonistic activities. By subjecting these cultures to close proximity, scientists can closely observe their interactions. They monitor several critical aspects, including growth inhibition, morphological changes and the production of inhibitory metabolites. The formulation and optimization of the consortium are given below in the form of a flow chart:

- Around 15 different organisms were selected and tested for their compatibility
- Among them, 4 compatible organisms (*Bacillus Spp, Pseudomonas Spp, Trichoderma spp, Penicillium Spp*) were selected for consortium formulation
- Each organism selected for the formulation was cultured separately on their respective growth

media (Nutrient media for bacterial species and PDA for fungal species)
- All the organisms were serially diluted and the concentration at 10^5 CFU was taken
- Each organism (10 mL of 10^5 CFU) was mixed in 500ml of minimal media (in 500 mL-2.5g of peptone+1 g of Dextrose)
- The prepared consortium was incubated for 48 hrs
- Then the consortium was tested for the presence of each organism by culturing them in selected media preferable for their growth
- After the confirmation of the presence of each organism in the consortium, it was further taken for the greenhouse trials

***Invitro* assay:** this assay was carried out by employing a well diffusion technique. The causative organism was spread across the plate, wells were bored. 10 µl of each potential antagonistic organism was added and incubated at room temperature for 48 hrs. After the incubation the zone of inhibition was measured and recorded in mm.

Green house evaluation of the microbial consortia:
Field trial design: The conduct of greenhouse trials in pomegranate orchards affected by bacterial blight disease represents a crucial step in evaluating the efficacy of microbial consortia as a biocontrol strategy. The trial design appears to be well-structured, aiming to assess the impact of these treatments on disease management. Here's an elaboration of the procedure:

Selection of affected orchards: Identify pomegranate orchards that are known to be affected by bacterial blight disease. Ensure these orchards are representative of the target conditions.

Trial design: Establish a clear trial design that includes different treatment groups. In this case the design consists of:

- **treatment plots:** These are plots where the microbial consortia are applied. The application rates and frequencies should follow recommended guidelines to ensure consistency
- **control plots:** These are plots where control substances are applied. Control substances may include water (to account for the effects of application without any active agents) or a standard pesticide (for benchmarking purposes)
- **Untreated control plots:** These plots receive no treatment and serve as a baseline for disease development in the absence of any intervention

Randomization and replication: Randomly assign the treatment groups to different sections of the orchard to minimize potential bias. Ensure an adequate number of replicates for each treatment group to improve statistical validity.

Application of treatments: Apply the microbial consortia, control substances (water or pesticide) and water (for untreated control plots) to their respective plots following recommended application rates and frequencies. This ensures that the treatments are applied consistently and accurately.

Disease assessment: Recording disease severity and incidence at regular intervals in both treated and control plots is a fundamental aspect of evaluating the efficacy of the microbial consortia as a biocontrol strategy for bacterial blight disease. Here's a more detailed explanation of this monitoring process.

Disease severity assessment: Disease severity refers to the extent or intensity of disease symptoms on the affected plants. Visual observations are made at predetermined intervals throughout the trial to evaluate how severe the disease symptoms are in both the treated and control plots.

Disease incidence assessment: Disease incidence measures the proportion of plants or plots that are affected by the disease. It indicates the spread or prevalence of the disease within the experimental area. Observations are made to determine whether plants become infected or remain disease-free over time. Percentage disease incidence are calculated using the below given formulae:

$$\text{Fruit incidence}(\%) = \frac{\text{Number of inf ected fruit}}{\text{Total fruit observed in a set}} \text{x} 100$$

$$\text{Leaf incidence}(\%) = \frac{\text{Number of inf ected leaves}}{\text{Total fruit observed in a set}} \text{x} 100$$

Visual observations: Visual observations involve inspecting individual pomegranate plants within each plot. Trained observers examine the plants

Table 1: Grade Ratings to record severity of the disease

	Percent incidence	
Grade	Leaves	Fruits
0	0.00	0.00
1	Up to 1	Up to 1
2	>1- 10	>1- 10
3	>10-20	>10-20
4	>20-40	>20-40
5	> 40-100	> 40-0
6	>70-100	

Table 2: Biochemical characterization of Xap isolate

Biochemical Tests	*Xap* isolates form fr Hosadurga
Gram's reaction	-
Starch hydrolysis	0
Indole production	0
Catalase test	0
KOH test	0
Gelatin liquefaction	0
Acid production	0

Table 3: Characterization of X. axonopodis pv. Punicae with specific marker genes 16sRNA and gyrB

Primers	Sequence (5'-3')	Annealing temperature (0C)	Size (bp)
16S rRNA	Xsp16SRFWD2-CTTACGCTAATACCGCATAT	55	761
	ACG Xsp16SREV1- CTGATCTGCGATTACTAGCGA		
	KKM5 Forward 5'GTTGATGCTGTT CACCAGCG3'	55	491
gyrB	KKM 6 Reverse 5'CATTCATTT CGCCCAAGCCC3'		

Table 4: Comparison of antagonistic activity of individual organisms in the consortium v/s consortium

Organism Name	Antagonistic activity in mm
Bacillus subtilis	15.2±0.185
Pseudomonas spp	9.9±0.121
Trichoderma spp	16.5±0.306
Penicillium spp	12.5±0.426
Microbial Consortium	25±0.121

Table 5: Green house efficacy of Microbial consortium against bacterial blight of pomegranate (per cent disease index on leaves and fruits)

No of days of spray	PDI of Leaves (in %)	PDI of fruits (in %)
5	15.32	17.15
10	20	22.37
15	22.35	30.17
20	28.72	26.23
25	21.81	18.72
30	09.52	08.67

Table 6: Comparison of weight of control fruits and test fruits

No of days of spray	Weight of control fruits (in grams)	Weight of test fruits
5	150	150
10	120	160
15	100	180
20	100	200
25	80	230
30	80	260

for characteristic symptoms of bacterial blight, which may include leaf lesions, wilting, canker formation, or other disease-related changes.

Rating scales: Rating scales are commonly used to standardize the assessment of disease severity and incidence. Observers assign scores or ratings based on predefined criteria. For example A severity scale might range from 0 (no symptoms) to 5 (severe symptoms covering a large portion of the plant). An incidence scale may represent the percentage of infected plants, such as 0% (no infection) to 100% (all plants infected) The severity of bacterial blight of pomegranate was recorded by using 0-5 and 0-6 scale on leaf and fruit respectively.

Results anddiscussion Isolation and identification of pathogen: Pomegranate bacterial blight causal organism was isolated from the Hosadurga region. Isolation was done by serial dilution and streak plate method. After obtaining separate colonies of each isolate, they were individually streaked onto Yeast-Dextrose-Calcium-Carbonate Agar medium (YDCA) and subsequently incubated at 30 °C for 72 hrs. Purified colonies from the YDCA medium were then carefully preserved at -20 °C in a nutrient broth and glycerol solution for future investigations. Fig 1 shows the isolated pure culture, Fig 2 is the microscopoic view of the pathogen and Fig 3 is the result of Gram staining showing pink rods.

Morphological characteristics: The pathogen was identified based on cultural and morphological characteristics (size, shape, texture and colony color). All the isolates differed in color, shape, size and appearance. The isolates, GP-2, GP-3, GP-6, GP-7, GP-10, GP-2, GP-4, GP-1, GP-2 and GP-3 showed light yellow colonies, while GP-1 and GP-4 showed ceramic yellow colonies and remaining isolates showed yellow color colonies on the medium. All the isolates showed small to medium and medium to large size, small circular and circular to irregularly shaped and highly raised, glistering and slightly raised glistering appearance on NA and YDCA. All the isolates were tested at Room temperature. The data clearly shows that this temperature was found optimum for the growth of the pathogen and the maximum number of colonies was observed. Notably, the colonies on the YDCA medium displayed characteristic features, appearing yellow, mucoid, slimy and raised, which aligns with the typical colony morphology associated with *Xanthomonas axonopodis pv. Punicae.*

Biochemical characterization: The bacterial isolates in question possess several distinctive characteristics. They are Gram-negative, indicating their cell wall structure. Additionally, they exhibit negative reactions for casein hydrolysis, phenylalanine production and the fermentation of lactose, maltose and D-sorbitol. Conversely, these isolates display positive traits such as starch hydrolysis and the ability to liquefy gelatin. They also produce hydrogen sulfide (H2S), possess catalase activity and show positive results in the oxidase test. Furthermore, they exhibit urease production and can undergo nitrogen reduction. Notably, these isolates can ferment various carbon sources, including glucose, fructose, sucrose, dextrose and dulcitol. Collectively, these characteristics are indicative of the bacterial isolates belonging to the species *Xanthomonas axonopodis pv. punicae.* The results of the same has been tabulated in (Table 1).

Molecular identification of pomegranate bacterial blight pathogen: The pathogen's genomic DNA was extracted using the Amnion Bacterial DNA Isolation Kit. To identify and amplify a specific region of the DNA, Polymerase Chain Reaction (PCR) was employed, utilizing universal primers targeting the 16S rRNA gene and specific primer for gyrb gene. The success of the

PCR amplification was confirmed through gel electrophoresis, which involved running the amplified DNA through a 1.2 percent gel. Notably, the gel electrophoresis displayed a distinct 761 base pair (bp) band corresponding to the 16S rRNA gene, confirming the presence and successful amplification of the desired DNA fragment. A primer set named KKM-5 Forward (5'GTTGATGCTGTT CACCAGCG3') and KKM-6 Reverse (5'CATTCATTT CGCCCAAGCCC3') as mentioned in Table 3 was designed based on the alignment analysis of the 530th C-terminus region of the gyrB genes. This primer set successfully amplified a DNA product with a size of 491 base pairs (bp), which was found to be specific to only *Xanthomonas axonopodis pv. punicae (Xap)*, Subsequently, the amplified DNA product was subjected to sequencing, a process carried out by the facilities of Amnion Biotech Private Limited, allowing for further analysis and identification of the pathogen.

Legend: M-Ladder 10 kb ladder 1,2,3,4-Xap isolates The PCR amplification of *Xanthomonas axonopodis pv. punicae* (Xap) using universal primers designed for the conserved 16S rRNA gene Fig 4 is a pivotal discovery with far-reaching implications in the field of plant pathology and diagnostics. This success underscores the specificity and reliability of universal primers, which are engineered to target highly conserved genetic regions found across diverse bacterial species. The detection of a 761 base pair (bp) band corresponding to the 16S rRNA gene in Xap signifies the presence of this pathogenic species within the sample, forming a foundational step in the precise identification and characterization of the causative agent responsible for pomegranate disease. Given the widespread utilization of the 16S rRNA gene as a molecular marker for phylogenetic investigations, this achievement validates its applicability in the context of Xap. Moreover, the remarkable specificity of the amplification reaction, which excluded other phytopathogenic Xanthomonads and related bacteria such as *X. axonopodis pv. citri, X. campestris pv. campestris, X. oryzae pv. oryzae, X. campestris mangiferae indicae and Pantoea agglomerans*, is of paramount importance. Fig 5 indicates the Gel run with gyrB amplified region. This being one of the specific region present in *Xanthomonas axonopodis pv punicae.* Formation of band at 491 bp confirms about he species identification. 3.3 16S rna Sequence analysis of isolated Xap. The analysis of the 16S rRNA gene sequence serves as a powerful tool for the precise identification of *Xanthomonas axonopodis pv. punicae* (Xap) and is a fundamental practice in the field of microbial taxonomy. Commencing with the acquisition of the 16S rRNA gene sequence from the unknown bacterial isolate, presumed to be Xap, the process unfolds through a series of critical steps. These include rigorous sequence quality control to ensure data accuracy and the subsequent alignment of the obtained sequence with publicly available 16S rRNA gene databases, such as the NCBI GenBank. The construction of a phylogenetic tree Fig 6 further elucidates the evolutionary relationships of the sequence, providing insights into its taxonomic placement. Subsequently, the sequence is scrutinized in comparison to reference strains and closely related *Xanthomonas* species, allowing for a determination of its affiliation.

Selection of microbial strains for the consortium: The process of selecting and evaluating 15 different organisms for their potential antagonistic activity against *Xanthomonas axonopodis pv. punicae* (Xap) is a crucial stage in the development of biocontrol strategies to combat bacterial blight in pomegranate orchards. This undertaking commenced with a thorough research review, where existing knowledge and literature on microorganisms with antagonistic properties against Xap were examined. This step aimed to identify promising candidates for further investigation. Subsequently, these 15 candidate organisms were procured in their pure form, ensuring their genetic and functional characteristics were well-defined and consistent for research purposes. With the organisms in hand, the critical phase of antagonistic activity screening followed. In the controlled environment of the laboratory, each organism was subjected to rigorous testing to assess its effectiveness in inhibiting the growth or pathogenic activity of Xap.
Out of this array of organisms, four strains namely *Bacillus Spp, Pseudomonas Spp, Trichoderma spp and Penicillium Spp* stood out as the most promising. They exhibited a notably higher level of antagonistic activity when compared to the other candidates. The selection of these superior strains was based on criteria that likely encompassed the extent of inhibition, consistency of performance and other relevant factors. These four chosen strains have showcased their potential as formidable biocontrol agents in combating Xap and are likely to be further investigated and developed for practical applications in managing bacterial blight in pomegranate cultivation.

Preparation of microbial consortia: To prepare the Microbial consortium, the selected four strains were first subcultured and maintained in their pure form. The next crucial step is to assess the compatibility of these strains to grow together harmoniously.

Screening the compatibility: Every chosen organism underwent compatibility testing through co-culturing with the remaining selected organisms. Figures

6,7,8,9,10,11 shows the Compatability results. None of the organisms inhibited the other's growth paving way for the further formulation.

Following plates the results of the co-culturing of the selected strains From the above results, it is evident that the selected strains did not inhibit the growth of other strains, allowing us to carry out the work further. Firstly, it confirms that the compatibility testing conducted on the selected strains has yielded favourable outcomes. This is of paramount importance in the further process of developing the consortium where the ability of different organisms to coexist and function together is of utmost importance and forms the basis of the study. The absence of growth inhibition among the selected strains signifies that they can thrive together in a cooperative manner. This opens up possibilities for further research and applications that require these strains to work synergistically. Furthermore, these results provided us with confidence in the feasibility of the experimental design. It suggests that the selected strains are compatible and can be utilized in future experiments, studies, or practical applications without concerns about antagonistic interactions that could compromise the desired outcomes. The confirmation of compatibility among the selected strains signifies a significant milestone in the research process, offering the assurance needed to progress with the work at hand, whether it involves scientific investigations, environmental solutions, or other areas where microbial interactions play a crucial.

***Invitro* assay:** Our experimental methodology was characterized by meticulous attention to detail, including the use of triplicate experiments, precise measurements and controlled incubation conditions. These measures were essential to obtain robust data on the antagonistic activity of the microbial consortium and the selected individual strains against *Xanthomonas axonopodis pv punicae.* (Fig 12-15) indicates the positive results of the antagonistic assay of individual strains against the Xap.

Consortium formulation: Following the optimization phase, we ultimately arrived at a conclusion wherein each organism was combined in a formulation consisting of 10 ml of 10^5 CFU (colony-forming units) for each and this mixture was diluted in 500 mL of minimal media. Subsequently, the formulated consortium underwent scrutiny to verify the presence of each selected organism. Upon confirming the presence of these strains, the consortium was subjected to in vitro testing to assess its antagonistic activity. A comparative analysis was conducted, evaluating the antagonistic potential of the individual strains against the formulated consortium. Upon achieving positive results, Fig 16a and Fig 16b the formulated consortium proceeded to field trials to determine its efficacy. Table 4 shows the tabulated values of Zone of inhibition formed by the individual organisms in the consortium and the developed Microbial Consortium. Graph 1 substantiate the results tabulated in (Table 4).

Statistical analysis: Each experiment was performed in triplets and the average mean and Standard deviation was calculated and recoded. One way ANOVA was performed and the significant difference was noted and recorded. From the performed ANOVA it is evident that there is significant difference in the values of antagonistic activity of selected organisms in the consortium and the antagonistic activity of the Microbial Consortium. Hence the hypothesis of Microbial consortium showing better results has been proved.

Green house trails: Throughout the 30-day trial period, while applying the formulated consortium, a keen eye was kept on the ongoing developments in the field. Visual observations played a crucial role in this process, allowing us to monitor any changes, improvements, or potential challenges that arose during this timeframe. In addition to these visual assessments, a systematic evaluation was conducted, employing a percentage disease index calculation method. This involved assigning specific ratings to various aspects of disease presence and severity simultaneously. By integrating these ratings, we were able to quantify the extent of disease impact accurately. This comprehensive approach enabled us to gain a holistic understanding of the consortium's effectiveness in mitigating disease and its overall performance under real-world field conditions. Such meticulous observations and data collection were instrumental in drawing meaningful conclusions about the practical applicability and efficacy of the formulated consortium.

The results of the 30 day trial period and the decrease in the percentage disease index are tabulated in Table 5. This shows that the greenhouse trails were positive in decreasing the pathogen effect on the plants. To substantiate the results from the trails Graphs 2 and 3 were plotted. The fruit yied was also considered as an observatory parameter to validate the formulated consortium. The results of the fruit yield of the treated and the untreated were observed and tabulated (Table 6) the same results were plotted (Fig. 4).

Conclusion and future work: Conducting field studies is essential to transition the promising findings of the developed microbial consortium from controlled lab and greenhouse settings to real-world agricultural applications. These studies provide a practical assessment of how the consortium functions in the

dynamic conditions of actual farms, considering various environmental factors and interactions. Field research enables the evaluation of long-term performance, ensuring the consortium's sustainability in disease control over multiple growing seasons. Moreover, it sheds light on how the consortium interacts with other microorganisms in the agricultural ecosystem, helping optimize its effectiveness. Lastly, field studies assess the economic viability, ensuring that the consortium offers a cost-effective and practical solution for pomegranate farmers grappling with blight disease. For Further studies, before getting the consortium to the market, the formulated Consortium has to be taken to Field trails to assess other climatic, environmental effects on its antagonistic action against the Xap.

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