International Journal of Bio-resource and Stress Management https://pphouse.org/ijbsm.php

Crossref

IJBSM July 2022, 13(7):683-692 Print ISSN 0976-3988 Online ISSN 0976-4038 **Article** AR2680

Research Article *Stress Management*

DOI: HTTPS://DOI.ORG/10.23910/1.2022.2680

Isolation and Characterization of Endophytes Against Bacterial Blight of Pomegranate

Monika Karn[1], Satish K. Sharma[1], Anju Sharma[2] and Joginder Pal[3]

[1]Dept. of Plant Pathology, [2]Dept. of Basic Sciences, Dr. YS Parmar University of Horticulture and Forestry, Nauni, Solan, Himachal Pradesh (173 230), India

[3]Dept. of Plant Pathology, CSK Himachal Pradesh Agriculture University, Palampur, Himachal Pradesh (176 062), India

Open Access

Corresponding karn.monika1990@gmail.com

0000-0002-5322-0491

ABSTRACT

The endophytic study was undertaken during 2019–2020 at department of plant pathology, Dr, YS Parmar UHF, Nauni, Solan (HP), India against *Xanthomonas axonopodis* pv. *punicae* inciting bacterial blight of pomegranate. Endophytes which we used, either endophytic bacteria or fungi have been isolated from four different genotypes/cultivars (Wild pomegranate, Kandhari, G-137 and Sindhuri) of pomegranate to study the isolation procedure and characterization of the endophytic microbes that colonize pomegranate plants. The huge diversity among the endophytic microbes and the host plants hinders a uniform protocol for isolation of endophytes and the establishment of inciting agent of bacterial blight. The most common technique that is currently used involves a thorough surface sterilization followed by fragmentation of the plant tissue and dilutions, culture of the fragments onto agar plates for endophytic bacteria and fragmentation of leaf bits after thorough surface sterilization onto agar plate for endophytic fungi. Among 27 and 19 bacterial and fungal endophytes, three potential microbes of each were selected after *in vitro* assay against *Xanthomonas axonopodis* pv. *punicae* which causes bacterial blight disease, and were identified based on morphological, biochemical, and cultural characteristics, respectively. This is the first report of endophytic microbes from the leaves of *Punica granatum* genotypes/cultivars in Himachal Pradesh, India. Future studies will determine the potential application of these isolates in biological control, growth promotion, enzyme production and colonization in host for induction of defense mechanism against bacterial blight disease of pomegranate.

KEYWORDS: Bacterial blight, endophyte, isolation, *in vitro* assay, pomegranate

***Citation* (VANCOUVER):** Karn et al., Isolation and Characterization of Endophytes Against Bacterial Blight of Pomegranate. *International Journal of Bio-resource and Stress Management,* 2022; 13(7), 683-692. HTTPS://DOI.ORG/10.23910/1.2022.2680.

Copyright: © 2022 Karn et al. This is an open access article that permits unrestricted use, distribution and reproduction in any medium after the author(s) and source are credited.

Data Availability Statement: Legal restrictions are imposed on the public sharing of raw data. However, authors have full right to transfer or share the data in raw form upon request subject to either meeting the conditions of the original consents and the original research study. Further, access of data needs to meet whether the user complies with the ethical and legal obligations as data controllers to allow for secondary use of the data outside of the original study.

Conflict of interests: The authors have declared that no conflict of interest exists.

RECEIVED on 15th October 2021 RECEIVED in revised form on 20th June 2022 ACCEPTED in final form on 16th July 2022 PUBLISHED on 31st July 2022

1. INTRODUCTION

Pomegranate is one of the most economically important fruit crops of India. Maharashtra region is the largest producer of pomegranate in the country. This fruit crop suffers heavily due to bacterial blight disease which is predominant in India. This disease hampered 70–80% crop production by reducing fruit quality and productivity (Sharma et al., 2009; Mondal and Singh, 2008). The bacterial blight was first reported in India from Rajasthan in 1952 (Hingorani and Mehta, 1952). Since 1998, bacterial blight disease has been appearing as a major production problem in important pomegranate growing states in India (Chavan et al., 2016). However, it received a minor importance earlier but appeared as a serious threat in all pomegranate growing regions of Maharashtra, Northern Karnataka and Andhra Pradesh (Yenjerappa et al., 2004). The disease could not be mitigated effectively due to rapid build-up of inoculums and spread of the disease widely. Many measures like biocontrol agents, combinations of antibiotics and pesticides along with phytochemicals and cultivation practices were not sufficient to remedy the problem (Manjula et al., 2002; Erayya et al., 2014). The phyllosphere is the habitat for a large diversity of microorganisms. Amongst them, a group of endophytic bacteria and fungi may colonize the internal tissues of host plants (Alves-Junior et al., 2021). These microbes can be isolated from flowers, fruits, leaves, stems, roots, and seeds of various plant species (Qin et al., 2011). Majority of them penetrate plants through the roots (Afzal et al., 2014). However, they may enter plants from the phyllosphere through the leaves, via stomata (Senthilkumar et al., 2011). They can be divided into two categories according to where they are found in the host: root endophytes and leaf endophytes (Ding and Melcher, 2016). The same endophytic microorganism can colonize several hosts (Agrios, 2005). Several studies have indexed isolates of endophytic microbes from different plant organs in various agricultural crops (Ryan et al., 2008; Miliute et al., 2015). Currently, the endophytes are appearing as potential tool in the field of agricultural biotechnology (Afzal et al., 2019). This interest relates to the results of several studies in which it was possible to observe the potential of microorganisms that grow in association with plants to promote their growth, due to their antagonism against phytopathogens (Etminani and Harighi, 2018; Young et al., 2013). If these microorganisms would to be applied in agricultural systems, they could be a novel alternatives, by accelerating sustainability and reducing the harmful effect of pesticides (Santos and Varalho, 2011). The interaction between host and endophytic microbes is not well understood. However, many isolates seem to have beneficial effects on their hosts (Ulrich et al., 2008). These beneficial effects include promoting host growth and biological control of phytopathogens (Costa et al., 2012). The population density of endophytic bacteria can vary from 10^2–10^9 (Overbeek and Elsas, 2008) and it depends on various factors, including the plant being studied, part under analyzation (QuadtHallmann and Klopper, 1996), developmental stage of the plant, plant cultivar (genotype) (Overbeek and Elsas, 2008), their interaction with other organisms, as well as other environmental-related factors (Hallmann et al., 1997). Considering the importance of fruit crop and seriousness of the disease, the present study was carried out to isolate, characterize and evaluate their potential under *in vitro* conditions for the management of *Xanthomonas axonopodis* pv. *punicae* in Himachal Pradesh.

2. MATERIALS AND METHODS

2.1. Sampling, isolation, purification and maintenance of bacterial and fungal endophytes

The healthy leaf samples were collected from four cultivars (Kandhari, Sindhuri, G-137 and wild pomegranate i.e. 'Daru') of pomegranate during 2019 from UHF, Nauni, Solan, immediately brought to the laboratory, and was used within an hour. The leaf samples were weighed to 1 g and washed under running tap water. They were then cut into small pieces (5 mm^2 dia) and then surface-sterilized with 70% ethanol and 4% sodium hypochlorite followed by rinsing in sterilized DW and were blot-dried using sterile filter paper. For isolation of bacterial endophytes, serial dilutions were made up to 10^{-5} dilutions. 100 µl from each dilution was poured in labelled (10^{-1}–10^{-5}) Petri plates containing nutrient agar medium, and spread with a spreader. For isolation of fungal endophytes, surface sterilized leaf bits were placed into Petri plates containing potato dextrose agar medium with help of sterilized forceps. The plating was done in triplicate and was incubated at 37°C for 72–96 h and 28±2°C for two weeks for the growth of bacterial and fungal endophytes, respectively. Further, isolated endophytes were revived and stored for preservation, on their respective medium (Pal, 2019; Kharwar et al., 2010).

2.2. Enumeration of bacterial and fungal endophytic population

Population of bacterial endophytes were enumerated by serial dilution (10^{-2}) and plate counting method. Colony forming unites were evaluated only after appropriate incubation at 37°C for 24 h (Pal, 2019). The colony count was performed in triplicate and was calculated as:

$$\text{Cfu ml}^{-1} = \frac{\text{Number of colonies} \times \text{Dilution factor}}{\text{Volume of sample taken (ml)}}$$

Periodically the bits were examined for the appearance of fungal colony after incubation, the fungal colonies were counted for individual species and the total number was enumerated. The colonization frequency (CF), expressed as percentage, was calculated according to Nalini et al. (2014) as follows:

$$\% \text{ CF} = \frac{\text{Number of tissue segments colonized by a fungus}}{\text{Total number of tissue segments plated}} \times 100$$

2.3. In vitro antagonism assay of bacterial and fungal endophytes

The bacterial and fungal endophytes isolated from pomegranate leaves were evaluated for their efficacy against the growth of Xap by inhibition zone assay method (Poovarasan et al., 2013). Observations were recorded for the zone of inhibition produced by them around the growth of the pathogen.

2.4. Morphological and biochemical characterization of bacterial endophytes

The different morphological traits of bacterial endophytes were analyzed includes: colony type, colony colour, its surface, margin as well as elevation of colony. Following biochemical tests were performed to ascertain the identity of the endophytes.

2.4.1. Gelatin liquefaction

Nutrient medium supplemented with 0.4 per cent gelatine was prepared in DW, and heating gently to dissolve. Dispense 2–3 ml of media into culture tubes, and then sterilize medium at 121°C (15psi) for 15minutes after that the cooled medium were stab-inoculated with bacterial culture, and incubated at 25°C for a longer period (7–14 days). Gram negative bacteria do not hydrolyse gelatine thus media remains solid.

2.4.2. Oxidase test

A disc of filter paper, impregnated with 1% aqueous solution of tetramethyl-p-phenylenediaminedihydrochloride was placed in a Petri dish on which a colony of freshly-grown bacterium was rubbed with the help of a sterile platinum wire (Kovacs, 1956). A dark, bluish-violet colour developed at the smear' within 10 seconds indicated positive assay. Negative or delayed (15–60 seconds) positive reaction indicated poor oxidase activity.

2.4.3. Potassium hydroxide test

A few drops of KOH (3%) were put on the slide. A loopful of 48 h old culture of bacterial suspension was smeared onto the KOH solution for 5–10 seconds. Pulled up the loop for identification of the pathogen. The bacteria formed a viscous slime, which reflected positive reaction.

2.4.4. Catalase test

The enzyme acts upon hydrogen peroxide, liberating oxygen and water. The test bacterium was grown on a slope of NGA, 1 ml of H_2O_2 (3%, 10 vol.) was poured over the culture. Formation of effervescence of gas bubbles indicated positive assay for catalase.

2.5.6. Nitrate reduction test

A disc of filter paper, impregnated with α-naphthylamine and sulphanilic acid was placed in a Petri dish on which a colony of freshly-grown bacterium was rubbed with the help of a sterile platinum wire. A distinct red or pink colour indicates nitrate reduction.

2.6. Morphological characterization of fungal endophytes

The isolated fungal endophytes were studied and identified on the basis of different morphological and cultural features of mycelial growth of the fungi. The mycelium of fungal endophytes was stained with lactophenol cotton blue for 10 minutes. Thereafter, stained microscopic slides were visualized at 40X magnification using compound microscopes.

2.6. Isolation of bacterial pathogen

Infected leaves showing characteristics symptoms were collected and brought to the laboratory for isolation and identification of the associated pathogen. A loopful of bacterial suspension was streaked on sterilized NGA plates and they were incubated at 28°C for 72 h and observed for colony development of the pathogen. The pathogen was identified on the basis of morphological, cultural, and biochemical characters as suggested by Dye (1978). Further culture was purified by streak plate method, and maintained on NGA slants at 4°C for further studies.

2.7. Statistical analysis

The data obtained from laboratory experiments were subjected to appropriate statistical analysis wherever necessary using standard procedure, as described by Gomez and Gomez (1984).

3. RESULTS AND DISCUSSION

3.1. Isolation and enumeration of endophytes from pomegranate leaves

The data embedded in table 1 revealed that leaf samples collected from cultivars of pomegranate (Wild pomegranate, Kandhari, G-137, and Sindhuri) at different locations of Nauni. The maximum bacterial endophytic population (57.21×10^3cfuml^{-1} leaves) was isolated from Kandhari cultivar (plate 1) of pomegranate followed by Wild pomegranate (47.54×10^3cfu ml^{-1} leaves). However, least endophytic bacterial population was observed in G-137 (10.37×10^3 cfu ml^{-1} leaves) followed by Sindhuri cultivar (4.03×10^3cfu ml^{-1} leaves). Similarly, maximum fungal endophytic population (6.57g^{-1} leaves) was isolated from cultivar G-137 and Sindhuri cultivar (6.24 g^{-1} leaves) (plate 2), whereas least population was isolated from Kandhari (4.37 g^{-1} leaves) followed by Wild pomegranate (3.18 g^{-1} leaves). It is documented in the literature that endophytes within different host plant species vary (Knief et al., 2010).

Table 1: Population of bacterial and fungal morphotypes obtained from different pomegranate genotypes

Genotype	Endophytes	
	Bacterial population (cfu ml^{-1})	Fungal population (CF%)
Wild pomegranate (Daru)	47.54	3.18
Kandhari	57.21	4.37
Sindhuri	4.03	6.24
G-137	10.37	6.57

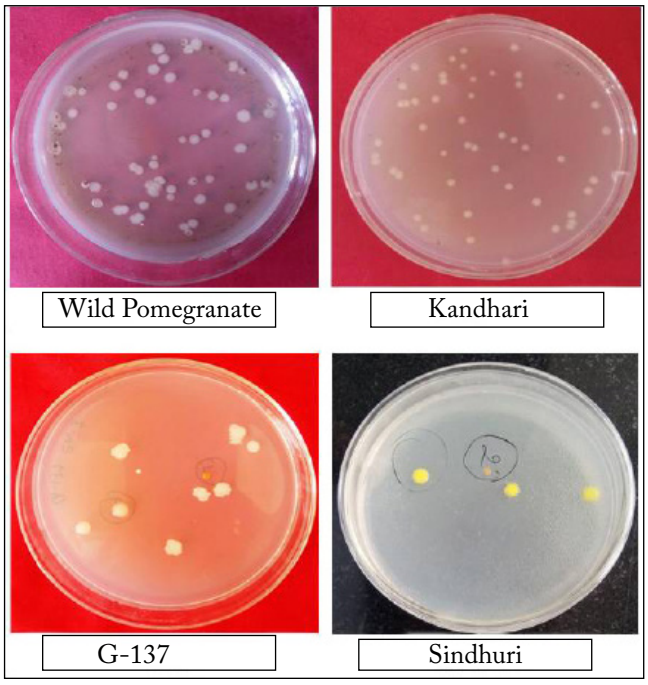

Plate 1: Endophytic bacterial population (10^2 efu m^{-1})isolated form different pomegranate genotypes/cultivars

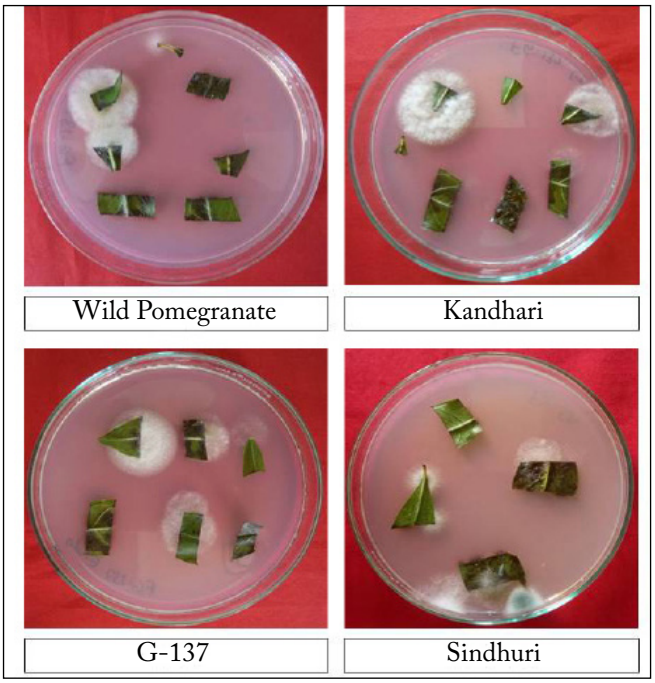

Plate 2: Endophytic fungal population isolated form different pomegranate genotypes or cultivars

Isolation of epiphytic and endophytic microbes yielded more number of bacteria than fungi. Tropical plant communities are rich in biodiversity including epiphytes and endophytes diversity in contrast to temperate regions. However, tropical epiphytes and endophytes are less host-specific than those from temperate areas.

3.2. Quantitative estimation of endophytes

Perusal of data presented in table 2 indicated that endophytic population varied to greater extent in the samples collected from wild pomegranate and different cultivars. A total of 46 endophytes were isolated from pomegranate leaves. Out of 46 isolates, 27 were endophytic bacteria and 19 were fungal endophytes. Among endophytes wild pomegranate (*Daru*) harboured more bacterial endophytes whereas higher number of fungal endophytes was isolated from G-137. The predominant bacterial and fungal endophytic colonies were selected, purified, and the pure culture of each isolate was maintained at 4°C on Nutrient and Potato Dextrose Agar, respectively for further studies. The variation in microbial population may be attributed to climatic conditions of

Table 2: Diversity of bacterial and fungal endophytes from different pomegranate genotypes

Genotype	Bacterial population	Fungal population	Total
Wild pomegranate	10	1	11
Kandhari	8	4	12
Sindhuri	2	3	5
G-137	7	11	18
Total	27	19	46

the location, age of plant, variety or cultivar type, time of sampling, and physico-chemical properties of soil.

Similarly, 60 bacterial isolates were obtained from the surface and inner tissues of different organs (leaves, twigs, and knots), from two olive cultivars of varying susceptibilities to olive knot disease (*Pseudomonas savastanoi* pv. *savastanoi*), and a total of 27 bacterial strains were able to significantly inhibit *Pseudomonas savastanoi*pv. *savastanoi* growth (Mina et al., 2020) suggesting that isolates from both cultivars may use different modes of action to protect host plant from *Pseudomonas savastanoi* pv. *savastanoi* infection and also noted that endophytes exhibit a lesser inhibition potential against *Pseudomonas savastanoi* pv. *savastanoi* than epiphytes, which makes epiphytes as the first layer of plant defense, comprising the most promising agents for Oak knot disease biocontrol (Hajek and Eilenberg, 2018). Isolates from cv. Cobrançosa could stimulate the immunity of host plant to indirectly combat *Pseudomonas savatanoi*pv. *savastanoi* invasions, while isolates from cv. Verdeal Transmontana seem to act directly against the pathogen.

3.3. Pathogenic potential of Xanthomonas axonopodis pv. *punicae*

Pathogenicity test revealed that symptoms of disease appeared within 7–14 days of inoculation. The Koch's postulates were proved by re-isolating the same bacterium from the diseased leaves and were confirmed to be the same bacterium (*X. axonopodis* pv. *punicae*) by comparing with original culture. The bacterium identity was confirmed through above mentioned biochemical tests. The bacterium was tested positive for KOH test and negative for Gram staining indicating the Gram-negative nature of bacterium. The bacteria were negative for nitrate reduction, positive for oxidase and gelatin liquefaction, thus establishing the identity of the bacterium as *Xanthomonas axonopodis* pv. *punicae* (Xap).

3.4. Evaluation of endophytes against X. axonopodis pv. punicae

The isolated endophytes were screened under *in vitro* by zone inhibition assay against bacterial blight of pomegranate pathogen, Xap.

3.4.1. In vitro antagonism assay of bacterial and fungal endophytes

Out of total 27 bacterial endophytes isolated, 3 isolates showed good anti-bacterial activity (Table 3, plate 3). The maximum growth inhibition of 2.31 cm dia was shown by *Bacillus anthracis* strain BDEn-1 followed by *Pantoea vagans* strain BGEn-3 (1.61 cm) and *Bacillus anthracis* strain BGEn-2 (1.32 cm), whereas no inhibition zone was found on the plate taken as control. Poovarasan et al. (2013) reported anti-bacterial activity of *Streptomyces* strain against Xap by agar diffusion method and found that *Streptomyces fradiae* exhibited the highest zone of inhibition (2.41 cm dia) followed by *S. Avermitilis* (2.31cm), *S. cinnamonensis* (2.22 cm), *S. canus* (2.0 cm) and non-Streptomyces actinomycetes (NSA) *Leifsoniapoae* (1.83 cm). From the 27 isolates that significantly inhibited *Pseudomonas savastanoi* pv. *savastanoi* causing olive knot disease, the antagonistic effect was found highest within *Pseudomonas*, *Bacillus* and *Alcaligenes* with inhibitions ranging from 15.8–85.2%. Both *Pseudomonas* and *Bacillus* have been reported to be the most promising biocontrol agents of several plant diseases, in particular the ones affecting roots

Table 3: *In vitro* antagonistic activity of bacterial and fungal endophytes against *X. axonopodis* pv. punicae

Isolate	Zone of inhibition (cm)
BDEn-1	2.31 (1.81±0.13)
BGEn-2	1.61 (1.60±0.14)
BGEn-3	1.32 (1.52±0.10)
FSEn-1	1.45 (1.56±0.11)
FGEn-2	1.58 (1.60±0.12)
FGEn-3	1.51 (1.58±0.10)
control	0.00 (1.00±0.00)
SEm±	0.11
CD (p=0.05)	(0.32)

Figures in parenthesis are square root transformed values

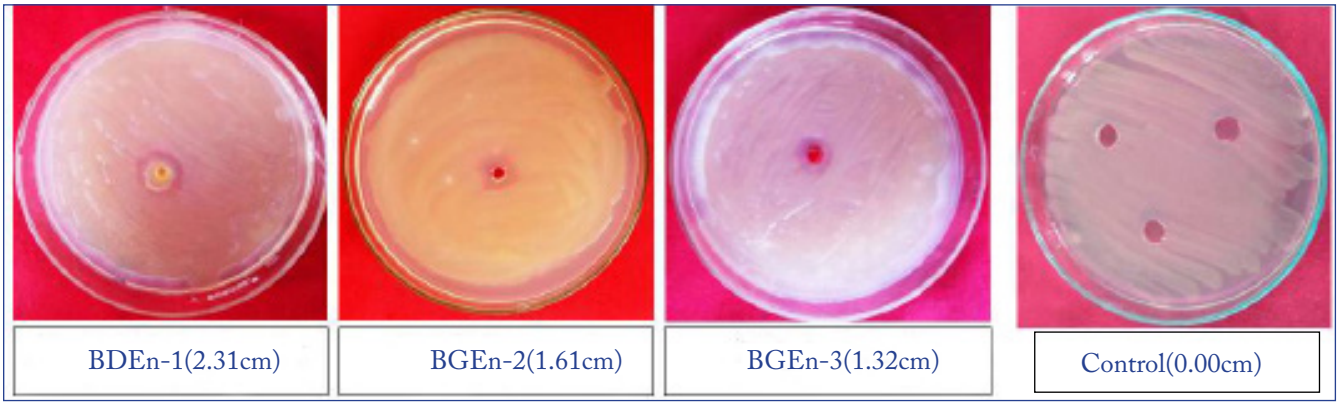

Plate 3: *In vitro* evaluation of bacterial endophytes against *Xanth omonas axonopodis* PV.punicae

(Mina et al., 2020). Munjal et al. (2015) isolated *Bacillus megatarium* (BmBP17) a root endophytes of black pepper were found effective against *Ralstonia solanacearum* and *Xanthomonas axonopodis* pv. *punicae*. Webster et al. (2020) documented medicinal plant endophytic isolates which showed antibacterial activity towards the plant pathogen, *Pectobactreium caratovorum* belonged to the genera *Bacillus*, *Klebsiella*, *Serratia* and *Enterobacter*. Out of total 19 fungal endophytes isolated, 3 isolates showed good anti-bacterial activity (Table 3, plate 4). The maximum growth inhibition of 1.58 cm dia was shown by *Albifimbria verucaria* strain FGEN-2 and *Trichoderma reesei* strain FGEn-3 (1.51 cm) followed by *Paradendryphiella arenaria*e strain FSEn-1 (1.45 cm), whereas no inhibition zone was found on the plate taken as control. Sornakili et al. (2020) isolated seven antagonistic fungal endophytes from rice leaves showing anti-fungal and anti-bacterial activities and identified as *Paecilomycestenuis*EF1, *Talaromyces*26 *pinophilus*EF2, *Nigrosporasphaerica*EF3, *Nigrosporaoryzae*EF4, *Trichoderma*27 *longibrachiatum*EF5,and EF7 and *Aspergillusterreus*EF6. Of these, *T. longibrachiatum*EF5 had the highest growth inhibition activity against the fungal plant pathogens, viz., *Macrphomina phaseolina*, *Magnoporthe grisea*, *Pythyium* sp., *Rhizoctonia solani, Fusarium oxysporum* and *Colletotrichum falcatum* (23%–82%) and bacterial pathogens viz., *Erwinia caratovora*, *Xanthomonas oryzae* pv. *oryzae* and *Ralstonia solanacearum* (13%–46%). An endophytic fungus, *Alternaria* GFAV15 with antimicrobial potential was isolated by Yadav et al. (2020) from the green unripe fruit of *Tinospora cordifolia*, showed strong antibacterial activity in combination against *Staphylococcus aureus* and *Escherichia coli*.

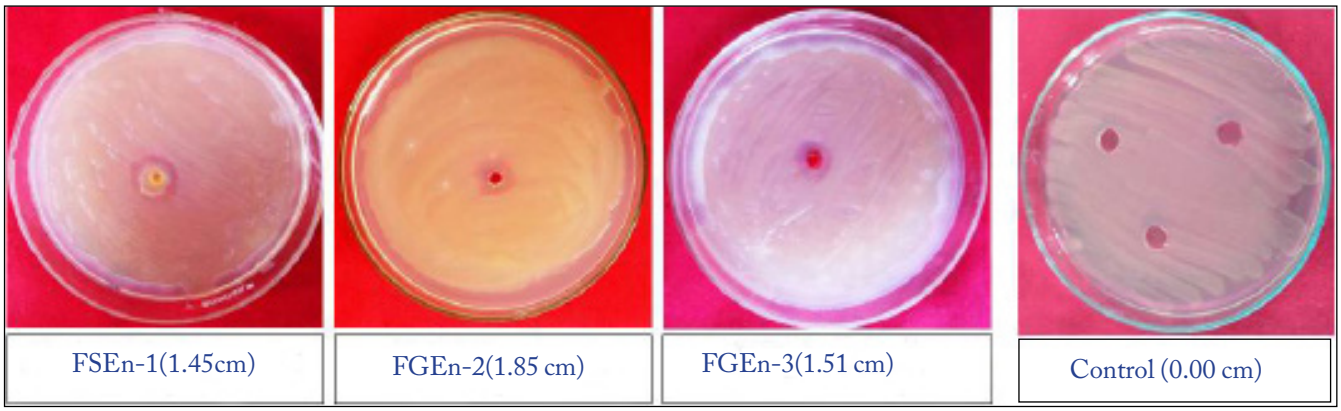

Plate 4: *In vitro* evaluation of bacterial endophytes against *Xanth omonas axonopodis* PV.punicae

3.5. Characterization of bacterial and fungal endophytes

3.5.1. Morphological and biochemical characterization of bacterial endophytes

All the selected bacterial endophytes showed variation in terms of their morphological, and biochemical characteristics (Table 4). The colonies of these isolates were small to medium, off-white to yellow, smooth, slightly raised or flat, and entire to wavy margin on nutrient agar plates. On the basis of Gram's reaction, out of 3 bacterial isolates 66.66% (2/3) were Gram positive, and 33.33% (1/3) were Gram negative. On the basis of biochemical characterization 100% (3/3) isolates were positive for oxidase and nitrate reduction, 66.66% for catalase production and gelatin liquefaction. Based on these morphological and biochemical characteristics of the representative isolates, and as per the identification keys given by earlier researchers to the different bacterial genera, the endophytic isolates BDEn-1,

Table 4: Morphological and biochemical characterization of bacterial endophytes

Sl. No.	Isolate	Morphological characters	Gram's reaction	Response to Biochemical Test			
		Colony morphology		Oxidase test	Nitrate test	Catalase test	Gelatin liquefaction
Endophytes							
1	BDEn-1	Medium, circular creamish white, smooth raised with wavy margin	Positive	+	+	+	+
2	BGEn-2	Medium, circular creamish white, smooth raised with wavy margin	Positive	+	+	-	+
3	BGEn-3	Small, punctiform, pale yellow, smooth, raised with entire margin	Negative	+	+	+	-

*+=Detected, - = Not detected

BGEn-2 were identified as *Bacillus* sp. (Osman and Yin, 2018; Sondang et al., 2019) and BGEn-3 was identified as *Pantoea* sp. (Vasebi et al., 2015; Singh et al., 2020).

3.5.2. Morphological and cultural characteristics of fungal endophytes

On the basis of *in vitro* antagonistic assay, most promising fungal endophytes were selected, and taken for morphological and cultural characterization. The data presented in table 5 revealed that colony colour of fungal endophytic isolates ranged from dark grey to white, fluffy to sparse or sub-merged mycelium with un-uniformed and slightly raised growth of mycelium (plate 5). The mycelium of all isolates

Table 5: Morphological and cultural characteristics of fungal endophytes

Isolate	Host	Culture colour	Mycelial characters	Sporulation	Mycelial growth rate (days taken to cover 90mm dia Petri plate at 26±2oC)
Endophytes					
FSEn-1	Sindhuri	Dark grey colour with un-uniform black margin	The hypae appeared septate and hyaline.	+	10
FGEn-2	G-137	Pure white in colour later randomly changed to olive green colour	The hypae appeared septate and hyaline. Cushioned like light to dark colouredsporodochia, 1-celled ovoid to elongate subhyaline to dark conidia.	+++	7
FGEn-3	G-137	Light white sparse mycelium later turn to green colour from centre	The hypae appeared septate and hyaline Conidiophores are hyaline, and branched. Conidia are hyaline, 1-celled, ovoid, borne in small terminal clusters, usually easily recognized by its rapid growth and green patches or cushions of conidia,	+	2

*+=Low sporulation, +++= Very high sporulation

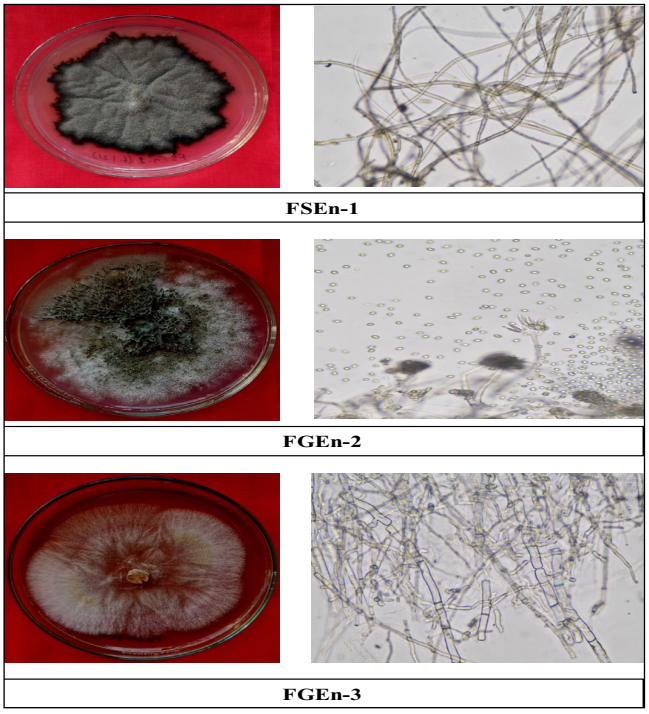

Plate 5: Morphological and microscopic characteristic of fungal leaf endophytes

was hyaline, and septate, however, significant variations have also been observed with respect to sporulation in different isolates. Isolate FGEn-2 had high sporulation, whereas isolates FSEn-1 had sparse sporulation and FGEn-3 had shown very less sporulation. Differences were also noticed in the colony growth rate as isolates took 2 to 10 days to fully cover the Petri plate. Based on these morphological and cultural characteristics of the representative isolates, and as per the identification keys provided by earlier researchers to the different fungal genera, the endophytic isolates FSEn-1 was identified as *Paradendryphiella* sp. (Liu et al., 2017, Orwa et al., 2020), FGEn-2 was identified as *Albifimbria* sp. (Rehman et al., 2021; Li et al., 2020; Gurung et al., 2019) and FGEn-3 was identified as *Trichoderma* sp. (Peciyulyte et al., 2014; Ahamed and Vermette, 2009).

4. CONCLUSION

This study demonstrated effectiveness of endophytic microbes isolated from pomegranate genotypes against *Xanthomonas axonopodis* pv. *punicae* through standardize isolation process for endophytic microbes under *in vitro* conditions. Among 27 and 19 bacterial and fungal endophytes, three potential microbes of each were selected

after *in vitro* assay against Xap, and were identified based on morphological, biochemical, and cultural characteristics, respectively. These findings also indicated the possible use of such endophytes as safe biological agents for management of bacterial blight of pomegranate.

5. ACKNOWLEDGEMENT

The authors are acknowledged to the Directorate of Research University of Horticulture and Forestry Nauni, Solan (H.P.), for conferring the laboratory facility at department of Plant Pathology for accomplishing this study.

6. REFERENCES

Afzal, I., Shinwari, Z.K., Sikandar, S., Shahzad, S., 2019. Plant beneficial endophytic bacteria: mechanisms, diversity, host range and genetic determinants. Microbiological Research 221, 36–49. doi: 10.1016/j.micres.2019.02.001 .

Afzal, M., Khan, Q.M., Sessitsch, A., 2014. Endophyticbacteria: prospects and applications for the phytoremediation of organic pollutants. Chemosphere 117, 232–242. doi: 10.1016/j.chemosphere.2014.06.078.

Agrios, G.N., 2005. Plant pathology (5thEdn.). Elsevier Academic Press, London, 952.

Ahamed, A., Vermette, P., 2009. Effect of culture medium composition on *Trichodermareesei's* morphology and cellulose production. Bioresource Technology 100, 5979–5987.

Alves-Junior, M., de Sousa, F.O., Silva, T.F., Albino, U.B., Garcia, M.G., Moreira, S.M.C.O., Vieira, M.R.S., 2021. Functional and morphological analysis of phylloplane and rhizoplane endophytic bacteria interacting in different cocoa production systems in the Amazon. Current Research in Microbial Sciences 2, 1–11.

Chavan, N.P., Pandey, R., Nawani, N., Nanda, R.K., Tandon, G.D., Khetmalas, M.B., 2016. Biocontrol potential of actinomycetes against *Xanthomonas axonopodis* pv.punicae, a causative agent for oily spot disease of pomegranate. BiocontrolScience and Technology 26, 351–372.

Costa, L.E.O., Queiroz, M.V., Borges, A.C., de Moraes, C.A., de Araujo, E.F., 2012. Isolation and characterization of endophytic bacteria isolated from the leaves of the common bean (*Phaseolus vulgaris*). Brazilian Journal of Microbiology, 1562–1575.

Ding, T., Melcher, U., 2016. Influences of plant species, season and location on leaf endophytic bacterial communities of non-cultivated plants. PLoS One 11. doi: 10.1371/journal.pone.0150895 .

Dye, D.W., 1978. Genus IX *Xanthomonas* Dowson 1939. In: Young, J.M., Dye, D.W., Bradbury, J.F., Panagopoulos, C,G., Robbs, C.F.A (Eds), *Proposed Nomenclature and Classification for Plant Pathogenic Bacteria*. New Zealand Journal of Agricultural Research 21, 153–77.

Erayya, L.R., Kumaranag, K.M., Chandrashekar, N., Khan, A.N., 2014. *In vivo* efficacy of some antibiotics against bacterial blight of pomegranate caused by*Xanthomonas axonopodis* pv. punicae. International Research Journal of Biological Sciences 3, 31–35.

Etminani, F., Harighi, B., 2018. Isolation and identification of endophytic bacteria with plant growth promoting activity and biocontrol potential from wild pistachio trees. Journal of Plant Pathology 34, 208–217. doi: 10.5423/PPJ.OA.07.2017.0158 .

Gomez, K.A., Gomez, A.A., 1984. Statistical procedures for agricultural research (2ndEdn.). John Wiley and Sons Inc, New York, USA. 680.

Gurung, S.K., Adhikari, M., Kim, S.W., Lee, H.G., Jun, J.H., Gwon, B.H., Lee, H.B., Lee, Y.S., 2019. First report of *Albi fimbria verrucaria* and *Deconica coprophila* (syn: *Psylo cybecoprophila*) from field soil in Korea. Korean Journal of Mycology 47, 209–218.

Hajek, A.E., Eilenberg, J., 2018. Natural enemies: An introduction to biological control (2nd Edn.). Cambridge University Press, Cambridge, UK.

Hallmann, J., Quadt-Halmann, A., Mahaffee, W.F., Kloepper, J.W., 1997. Bacterial endophytes in agricultural crops. Canadian Journal of Microbiology 43, 895–914.

Hingorani, M.K., Mehta, P.P., 1952. Bacterial leaf spot of pomegranate. IndianPhytopathology 5, 55–56.

Kharwar, R.N., Gond, S.K., Kumar, A., Mishra, A., 2010. A comparative study of endophytic and epiphytic fungal association with leaf of *Eucalyptus citriodora* Hook., and their antimicrobial activity. World Journal of Microbiology and Biotechnology 26, 1941–1948.

Knief, C., Ramette, A., Frances, L., Alonso-Blanco, C., Vorholt, J.A., 2010. Site and plant species are important determinants of the *Methylobacterium* community composition in the plant phyllosphere. Microbial Population and Community Ecology 4, 719–728.

Kovacs, N., 1956. Identification of *Pseudomonas pyocyanea* by the oxidase reaction. Nature 178, 703.

Li, Z., Chang, P., Gao, L., Wang, X., 2020. The endophytic fungus *Albi fimbria verrucaria* from wild grape as an antagonist of *Botrytis cinerea* and other grape pathogens. Phytopathology 110, 843–850.

Liu, N.G., Hongsanan, S., Yang, J., Lin, C.G., Bhat, D.J., Liu, J.K., Jumpathong, J., Boonmee, S., Hyde, K.D., Liu, Z.Y., 2017. *Dendryphiella fasciculata* sp. *nov.*and

notes on other *Dendryphiella* species. Mycosphere 8, 1575-1586.

Munjal, C.P., Khan, A.N.A., Ravikumar, M.R., 2002. Management of bacterial blight of pomegranate (*Punica granatum* L.) caused by *Xanthomonas axonopodis* pv. *punicae*. In: Annual Symposium Meeting on Plant Disease Scenario in Southern India, India.

Miliute, I., Buzaite, O., Baniulis, D., Stanys, V., 2015. Bacterial endophytes in agricultural crops and their role in stress tolerance: a review. Zemdirbyste Agriculture 102, 465–478. doi: 10.13080/z-a.2015.102.060 .

Mina, D., Pereira, J.A., Lino-Neto, T., Baptista, P., 2020. Screening the olive tree phyllosphere: search and find potential antagonists against *Pseudomonas savastoni* pv. *savastoni*. Frontiers in Microbiology 11, 1–12.

Mondal, K.K., Singh, D., 2008. Bacterial blight of pomegranatea technical bulletin. In: Division of Plant Pathology, IARI,New Delhi, India.

Munjal, V., Nadakkakath, A.V., Sheoran, N., Kundu, A., Venugopal, V., Subaharan, K., Rajamma, S., Eapen, S.J., Kumar, A., 2015. Genotyping and identification of broad spectrum antimicrobial volatiles in black pepper root endophyticbiocontrol agent, *Bacillus megaterium* BP17.Biological Control 92, 66–76.

Nalini, M.S., Ningaraju, S., Prakash, H.S., 2014. Endophytic fungal diversity in medicinal plants of western ghats, India. International Journal of Biodiversity 11, 1–9.

Orwa, P., Mugambi, G., Wekesa, V., Mwirichia, R., 2020. Isolation of haloalkaliphilic fungi from lakeMagadi in Kenya. Heliyon 6, 02823.

Osman, N.I., Yin, S., 2018. Isolation and characterization of pea plant (*Pisumsativum* L.) growth-promoting rhizobacteria. African Journal of Microbiology Research 12, 820–828.

Overbeek, L.V.,Elsas, J.D.V., 2008. Effects of plant genotype and growth stage on the structure of bacterial communities associated with potato (*Solanum tuberosum* L.).FEMS Microbiology Ecology64, 283–296.

Pal, J., 2019. Studies on management of white root rot (*Dematophora necatrix* Hartig) of apple in Himachal Pradesh. Ph.D. Thesis. Department of Plant Pathology. YS Parmar University of Horticulture and Forestry. Solan. 33p.

Peciulyte, A., Anasontzis, G.E., Karlstrom, K., Larsson, P.T., Olsson, A., 2014. Morphology and enzyme production of *Trichoderma reesei* Rut C-30 are affected by the physical and structural characteristics of cellulosic substrates. Fungal Genetics and Biology 72, 64–72.

Poovarasan, S., Mohandas, S., Paneerselvam, P., Saritha, B., Ajay, K.M., 2013. Mycorrhizae colonizing actinomycetes promote plant growth and control bacterial blight of pomegranate (*Punica egranatum* L. cv. Bhagwa). Crop Protection 53, 175–181.

Qin, S., Xing, K., Jiang, J.H., Xu, L.H., Li, W.J., 2011. Biodiversity, bioactive natural products and biotechnological potential of plant-associated endophyticactino bacteria. Applied Microbiology and Biotechnology 89, 457–473.

QuadtHallmann, A., Kloepper, J.W., 1996. Immunological detection and localization of the cotton endophyte *Enterobacter asburiae* JM22 in different plant species. Canadian Journal of Microbiology 42, 1144–1154.

Rehman, A., Mehboob, S., Alam, M.W., Naz, S., 2021. First report of leaf spot caused by *Albifimbriaverrucaria* on spinach in Pakistan. Journal of Plant Pathology 103, 715.

Ryan, R.P., Germaine, K., Franks, A., Ryan, D.J., Dowling, D.N., 2008. Bacterial endophytes: recent developments and applications. FEMS Microbiology Letters 278, 1–9. Doi: 10.1111/j.1574-6968.2007.00918.x .

Santos, T.T., Varallo, M.A., 2011. Application endophytic microorganisms in agriculture and production of substances of economic interest. Ciências Biologicas e da Saude 32, 199–212. doi: 10.5433/1679-0367.2011v32n2p199.

Senthilkumar, M., Anandham, R., Madhaiyan, M., Venkateswaran, V., Sa, T., 2011. Endophytic 895 bacteria: perspectives and applications in agricultural crop production. In: Bacteria in Agrobiology: Crop 896 Ecosystems. Springer, 61-96. doi: 10.1007/978-3-642-18357-7_3 .

Sharma, K.K., Jadhav, V.T., Sharma, J., 2009. Present status of pomegranate bacterial blight caused by *Xanthomonas axonopodis* pv. *punicae* and its management. In: II International Symposium on Pomegranate and Minor-including Mediterranean-Fruits, India. ISHS ActaHorticulturae 890.

Singh, D., Yadav, D.K.,Fatime, F., 2020. Characterization and genetic diversity of *Pantoea agglomerans* isolates having dual potentiality to suppress growth of *Ralstonia solanacearum* and plant growth promoting ability. Indian Phytopathology 73, 55–67.

Sondang, Y., Anty, K.,Siregar, R., 2019. Identification of endophytic and rhizosphere bacteria in maize (*Zea maydis* L.) in Limapuluh Kota region, west Sumatra, Indonesia. Earth and Environmental Science 347, 012002.

Sornakili, A., Thankappan, S., Palanisamy, S., Nithya, P., Uthandi, S., 2020. Antagonistic fungal endophytes and their metabolite mediated interactions against phytopathogens in rice. Physiological and Molecular Plant Pathology 112, 101525.

Ulrich, K., Ulrich, A., Ewald, D., 2008. Diversity of

endophytic bacterial communities in poplar grown under field conditions. FEMS Microbiology Ecology 63, 169–180.

Vasebi, Y., Alizadeh, A., Safaie, N., 2015. *Pantoea agglomerans* ENA1 as a biocontrol agent of *Macrophomina phaseolina* and growth enhancer of soybean. Journal of Crop Protection 4, 43–57.

Webster, G., Mullins, A.J., Cunningham-Oakes, E., Renganathan, A., Aswathanarayan, J.B., Mahenthiralingam, E., Vittal, R.R., 2020. Culturable diversity of bacterial endophytes associated with medicinal plants of the western ghats, India. FEMS Microbiology Ecology 96, fiaa147.

Yadav, V., Singh, A., Mathur, N., Yadav, R., 2020. Isolation and characterization of *Alternaria* GFAV15, an endophytic fungus from green fruit of *Tinospora cordifolia* (Willd.) Miers from semi-arid region. South African Journal of Botany 134, 343–348.

Yenjerappa, S.T., Ravikumar, M.R., Jawadagi, R.S., Khan, N.A., 2004. *In vitro* and *in vivo* efficacy of bactericides against bacterial blight of pomegranate. In: Proceedings of National Symposium of Crop Surveillance: Disease Forecasting and Management, New Delhi, India.

Young, L.S., Hameed, A., Peng, S.Y., Shan, Y.H., Wu, S.P., 2013. Endophytic establishment of the soil isolate *Burkholderia* sp. CC-Al74 enhances growth and P-utilization rate in maize (*Zea mays* L.). Applied Soil Ecology 66, 40–47. doi: 10.1016/j.apsoil.2013.02.001.