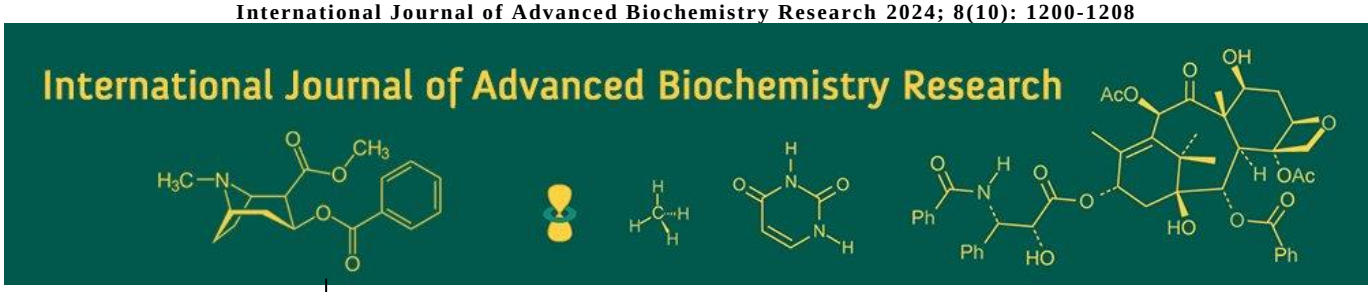

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Harnessing novel endophytes to combat potato common scab

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Abstract
Potato is cultivated all around the world and it is poor man's food. Marketed and economical value of potatoes deteriorates due to several abiotic and biotic stresses, among this common scab is major ones. Common scab is caused by various *Streptomyces* species, but major one is *Streptomyces scabies*. This pathogen can be managed by various biological as well as chemical treatments. In biological control endophyte is a major one, so isolation of pathogen and efficacy was checked. A total of 20 endophytic isolates were successfully obtained and screened for antagonistic efficacy against *S. scabies*. Five efficient endophyte bacterial isolates tested, PET-19 (*Alcaligenes aquatilis*), PER-17 (*Bacillus altitudinis*) and PER-2 (*Serratia marcescens*) showed the highest antagonistic activity.

Keywords: Common scab, *Streptomyces scabies*, endophytes, biochemical tests

1. Introduction
Common scab, caused by *Streptomyces* spp., is a significant soil-borne disease affecting potatoes (*Solanum tuberosum* L.), resulting in substantial economic losses globally. The disease manifests as corky lesions on the tuber surface, diminishing both market value and consumer acceptability (Kapuria *et al*., 2016) [6]. Traditionally, chemical control methods have been employed to mitigate this disease, often with limited success and accompanied by environmental concerns. Recently, the exploration of endophytes as a biological control measure has gained momentum. Endophytes are microorganisms, including bacteria and fungi, that live symbiotically within plant tissues (Kobayashi *et al*. 2012) [7]. They often confer benefits to their host plants, such as improved growth, stress tolerance, and resistance to pathogens (Kang *et al*., 2022) [5]. Integrating endophytic management strategies into agricultural practices offers a sustainable and eco-friendly approach to controlling the common scab of potatoes. This paper discusses the management of common scab through biological means and highlights the emerging potential of endophytes as biocontrol agents.

2. Materials and Methods
2.1 Isolation and evaluation of bacterial endophytes against *Streptomyces scabies*
2.1.1 Isolation of endophytic bacteria obtained from potato
Samples from healthy plant parts were collected from the Anand and Kheda districts of Gujarat and brought to the laboratory for the isolation of endophytic bacteria. Different plant parts *viz*., leaves, roots and tubers separated from the main potato plants.
Healthy leaves, roots and tubers were washed with water to remove the dust and thereafter plant parts were chopped into pieces of 4-6 mm. The disinfection and isolation were done as per the procedure of Araujo *et al*. (2001) [2]. Briefly, the plant parts were disinfected superficially by following the five-step protocol as 70 per cent alcohol for 1 minute, 4 per cent sodium hypochlorite for 15 sec and finally 3 rinses in sterile distilled water. To confirm the disinfection protocol, aliquots of the sterile water used in the final rinse and plated on NA medium at 28 °C for 2 days. After 2 days plates were examined for the presence or absence of bacterial colonies.
Another method was also used for the isolation of endophytes. Two-gram surface sterilized plant parts were macerated with 6 ml of 0.9% NaCl in a sterilized mortar and pestle. The macerated tissues were diluted serially in sterile distilled water. From this, 6 dilutions (10^{-1} to 10^{-6}) were made by transferring 1 ml of the suspension to successive sterile water columns.

From the last two series of the dilution, 0.1 ml was taken and plated onto the NA medium. The plates were incubated at 28 °C for 1–10 days until growth was observed.

2.1.2 Evaluation of endophytic bacteria against *Streptomyces scabies*

The antagonistic efficacy of endophytic bacterial isolates against *S. scabies* was evaluated by using the agar well diffusion method at PG Research Laboratory, Department of Plant Pathology, BACA, AAU, Anand by completely randomized design.

Observation recorded

Observation on the inhibition zone (mm) was recorded after 72 hrs of incubation. The per cent growth inhibition over control was calculated by using the formula given by Vincent (1947).

$$PGI = \frac{DC - DT}{DC} \times 100$$

Where, PGI = Growth inhibition (%), DC = Colony diameter in control (mm), DT = Colony diameter in respective treatment (mm)

2.1.3 Methodology

One ml of *S. scabies* culture suspension obtained from 72 hrs old culture was added to 20 ml of Nutrient agar medium and inoculated on a pre-sterilized Petri plate. After that media were spread evenly with an L shaped spreader. After solidification, four wells were formed with the help of a 5 mm diameter cork borer in a solidified Petri plate and 10 µl of each endophytic bacterial isolate was poured into the wells created on the medium. Inoculated Petri plates were incubated at room temperatures (28±2 °C) for 48 hrs and the diameter of the inhibition zone was measured after 72 hrs.

2.1.4 Molecular identification of endophytic bacteria

Identification of promising bacterial endophytes done by marker-assisted identification using 16S rDNA. DNA extraction of the endophytic bacteria was done by the conventional CTAB method and DNA was amplified with the 16S rDNA. The bacterial genomic DNA isolated from the individual endophytic bacterial culture was selected for 16S rDNA. PCR to amplify at 46- 56 ºC temperature gradient with the 16S ribosomal gene. The primer sequences are given below: 27 F- (5'-AGAGTTTGATCACTGGCTCAG -3′) and 1492 R(5′-TACGGACTTACCTTGTTACGACTT -3′). The aliquots were checked for amplification of the expected size on 1.8 per cent agarose gel and visualized through the gel documentation system. Then the PCR product of the potential endophyte was sequenced at Centre of Excellence in Biotechnology, AAU, Anand.

2.1.5 Morphological and biochemical characterization of potential endophytic bacterial isolates

Gram staining: Gram staining was performed as described by Aneja (2007) [1]. For Gram staining crystal violet, iodine, alcohol and safranin were used. At first, the isolated bacterial culture was fixed onto a glass slide by providing heat. Gently flood the smear with crystal violet and let stand for 30 seconds. Tilt the slide slightly and gently rinse with tap water or distilled water using a wash bottle. Gently flood the smear with Gram's iodine and let stand for 1 minute. Tilt the slide slightly and gently rinse with tap water or distilled water using a wash bottle. Apply the decolorizing agent drop by drop and immediately rinse with water. Gently flood with safranin to counter-stain and let stand for 30 seconds. Rinse the slide with water and air dry it. Finally, the slide was observed under a 100X microscope along with one drop of immersion oil. Positive and negative results were considered based on color changes as purple and pink, respectively.

Hydrogen cyanide (HCN) production test: HCN production of efficient endophytic bacterial isolates were tested qualitatively. The endophytic bacterial isolates were streaked on King's B medium amended with glycine. Sterile filter paper saturated with picric acid solution was placed on the upper lid of the Petri plate. The Petri plates were sealed with parafilm and incubated at 28 °C for 48 h and kept for observation.

Ammonia production test: Endophytic bacterial isolates were tested for the production of ammonia in peptone water. Freshly grown endophytic bacterial isolates were inoculated into 10 ml of peptone water in each test tube and incubated at 28 °C for 48 hrs. After 48 hrs. Nessler's reagent (0.5 ml) was added to each tube and observed for the development of a faint yellow to dark brown color as a positive indication of ammonia production.

Phosphate solubilization test: Endophytic bacterial isolates were screened for their potential to solubilize insoluble calcium phosphate on Pikovskaya agar medium as described by Pikovskaya (1948) [9]. The bacterial colony was placed in the center of Pikovskaya agar medium plates and incubated at 28±2 °C for 7 days. The phosphate solubilizing efficiency was measured based on halo zones around the colonies.

Proteolytic activity test: Proteolytic activity was tested by inoculation of 10 µl into 6 mm wells made on skim milk agar medium (containing 5 g pancreatic digest of casein, 2.5 g yeast extract, 1 g glucose, 15 g agar and 100 ml of 7 per cent skim milk solution per liter and incubated at 28 °C up to 4 days and observed for the formation of the halo zone around the colonies and a clear zone around the cells indicated positive proteolytic activity.

2.2 Statistical analysis

The data obtained from present investigations were subjected to statistical analysis by making use of the analysis of variance technique (Steel and Torrie, 1980). The standard methods of analysis of variance for completely randomized design and correlation were used in the experiments.

3. Results

3.1 Isolation and evaluation of bacterial endophytes against *Streptomyces scabies*

3.1.1 Isolation of endophytic bacteria obtained from potato

A total of 20 endophytic bacterial isolates were successfully obtained from various healthy plant parts of potatoes using the procedure outlined in 2.1.1. Among these isolates, 11 were from roots, 6 from leaves and 3 from tubers. (Table 1).

3.1.2 Screening of isolated endophytic bacteria against *S. scabies*

The study aimed to screen the antagonistic efficacy of endophytic bacteria isolated from healthy potato plant parts against *S. scabies* by agar well diffusion method as per the procedure given in 2.1.2 and observed the results as per Table 2.

3.1.3 Effect of efficient endophytic bacteria against *S. scabies*

The study aimed to evaluate the antagonistic efficacy of primarily screened endophytic bacteria isolated from healthy potatoes against *S. scabies*. Total five efficient endophytes obtained (Plate 1). The antagonistic efficacy of endophytic bacterial isolates was assayed *in-vitro* by the agar well diffusion method. The efficacy of the screened endophytic bacterial isolates to inhibit the growth of the pathogen was recorded in terms of inhibition zone diameter (mm). A total of five bacterial endophyte isolates were evaluated for their antagonistic efficacy against *S. scabies*. Among them, PET-19, PER-17 and PER-2 isolates showed the highest antagonistic efficacy with an inhibition zone of 29.50 mm, 27.25 mm and 20.50 mm, respectively (Table 3 and Plate 2). These findings are consistent with those reported by Shi *et al*. (2021), who collected endophytic bacteria from different locations in Jiaozhou city, China. They focused on roots, stems and tubers for isolation, identifying a total of 1,897 endophytic bacteria. Among these, 983 OTUs were found in roots, 561 OTUs in stems and 353 OTUs in tubers.

The endophytic bacterial isolates that showed the highest inhibition zone were considered as potential and studied for morphological, molecular and biochemical characterization.

3.1.4 Molecular identification of endophytic bacteria

The promising endophytic bacterial isolates that exhibited inhibition zones were identified as potential isolates and subjected to molecular characterization. Genomic DNA from these isolates was extracted following the CTAB method. The quality of DNA was assessed by running 2 µl of each sample mixed with 1 µl of gel loading dye on a 0.8 per cent agarose gel, while DNA quantity was measured using a spectrophotometer (Nanodrop Technologies). A spectrophotometer ratio of A_{260}/A_{280} ranging from 1.8 to 2.0 indicated the purity of the isolated DNA from the endophytic bacteria, confirming suitability for PCR amplification (Table 4). The DNA concentration was standardized to 50 ng/µl for subsequent 16S rDNA amplification and amplified product was observed at 1.8 per cent agarose gel (Plate 3).

3.1.5 Identification of potential antagonistic endophytic isolate based on sequence analysis of 16S rDNA

The PCR product was sequenced at the Centre of Excellence in Biotechnology, AAU, Anand. The 16S rRNA gene sequence of the selected isolate was compared with the 16S rRNA gene sequence available in the NCBI database through BLASTN search. The 16S rRNA gene sequences are widely employed for studying bacterial phylogeny and taxonomy due to their status as a primary genetic marker. This gene is part of the major rRNA and includes highly conserved regions across biological species, enabling comparative analyses in studies of molecular evolution. (Sharma *et al*., 2017) [11]. PET-19 isolated from the tuber of potato was identified as *Alcaligenes aquatilis* (GenBank Accession No., PP968187), PER-17 isolated from the root was identified as *Bacillus altitudinis* (GenBank Accession No., PP968180) and PER-2 isolated from root was identified as *Serratia marcescens* (GenBank Accession No., PP968398).

Li *et al*. (2019) identified the endophytic strain AMCC 101304 belonged to genus *Bacillus altitudinis*. In the pot experiments, the treatment of the pathogen disease grade was found to be significantly lower after treatment with *S. scabies* combined with the strain *B. altitudinis* AMCC 101304. Queiroz and Melo (2006) isolated endophytic strain R 3.5, which was identified as *Serratia marcescens*. They tested *S. marcescens* for control of *Phytophthora parasitica* towards citrus in greenhouse trials. The bacterium reduced the seedling infection in 50 per cent seedlings. Gong *et al*. (2019) [4] investigated 16S rRNA sequence of strain N1-4. The results indicated that sequences of N1-4 showed great similarity to the species of *Alcaligenes aquatilis*.

3.1.6 Morphological and biochemical characterization of potential endophytic bacterial isolates

The Endophytic bacterial isolates that showed efficient inhibition zones were considered as a potential and studied for morphological and biochemical characterization *viz.*, Gram's staining, HCN production, ammonia production, phosphate solubilization and protease production.

Gram's staining

Among the five isolates of endophytic bacteria, all isolates were Gram-negative, except PET-17. In Gram's staining test PER-19, PER-2, PEL-10 and PER-14 bacterial isolates failed to retain the violet color of the primary stain (crystal violet) and showed a reddish pink color which confirmed the Gram-negative characteristics of the isolated bacteria. Only PET-17 retains the violet color that confirms the Gram-positive nature of bacteria. All isolates were with rod-shaped cells (Plate 4). The morphological characteristics of the isolates revealed the dominance of Gram-negative bacteria over the Gram-positive bacteria Table 5.

An earlier study reported a predominance of Gram-negative bacteria by Elbeltagy *et al*. (2000) [3]. They morphologically characterized endophytic bacteria by gram staining. Out of 28 isolates, Gram negative isolates were 20 and only 8 isolates were Gram positive. Similar results were observed by Yousefi *et al.* (2018) [15].

Hydrogen cyanide (HCN) production test

Among all the isolates PET-19 and PER-2 showed a strong reaction (+++) of HCN production while isolate PEL-10 showed a moderate reaction (++) of HCN production (Table 6). PER-14 and PER-17 showed weak reactions by exhibiting slight change (-) in color from yellow to brown (Table 6, Plate 5).

Ammonia production test

All the efficient endophytic bacterial isolates were tested for Ammonia production (Plate 6). All the isolates produced strong (+++) dark brown color showing significant ammonia production (Table 6, Plate 6).

Phosphate solubilisation test

All the five endophytic bacterial isolates were tested for their phosphate solubilizing activity on Pikovskaya's agar medium (Table 7). The highest phosphate solubilization of

6.8 mm was shown by PER-2 isolate, while the no phosphate solubilization was shown by PEL-10 (Plate 7).

Protease activity test

Five endophytic bacterial isolates were evaluated for protease production. The formation of a clear halo zone around the bacterial growth on skim milk agar indicated significant production of protease. Among the five endophytic bacterial isolates, PET-19 isolate showed a halo zone of diameter 6.05 mm after 5 days of incubation while there was no zone observed in the PEL-10 and PER-14 (Table 7, Plate 8).

Table 1: Endophytic bacteria isolates from potato plant

Plant parts collected	Designated isolates	Total no. of isolates
Roots	PER-1, PER-2, PER-4, PER-7, PER-8, PER-12, PER-14, PER-15, PER-17, PER-18, PER-20	11
Leaves	PEL-3, PEL-10, PEL-11, PEL-13, PEL-16, PEL-9	6
Tubers	PET-5, PET-6, PET-19	3

Table 2: Screening of isolated endophytic bacteria against *S. scabies*

Sr. No.	Endophytic bacterial isolate	Inhibition zone	Sr. No	Endophytic bacterial isolate	Inhibition zone
1	PER-1	-	11	PEL-11	-
2	PER-2	+	12	PER-12	-
3	PEL -3	-	13	PEL-13	-
4	PER -4	-	14	PER-14	+
5	PET -5	-	15	PER-15	-
6	PET -6	-	16	PEL-16	-
7	PER-7	-	17	PER-17	+
8	PER-8	-	18	PER-18	-
9	PEL-9	-	19	PET-19	+
10	PEL-10	+	20	PER-20	-

Note: (1) + indicates the presence of the inhibition zone, - indicates the absence of inhibition zone
(2) PER: Potato endophyte from roots, PEL: Potato endophyte from leaves, PET: Potato endophyte from tubers,

Table 3: Effect of efficient endophytic bacteria against *S. scabies* under *in-vitro*

Sr. No.	Endophytic bacetrial isolate	Zone of inhibition (mm)	Inhibition over control (%)
1	PET-19	29.50	32.77
2	PER-17	27.25	30.22
3	PER-2	20.50	22.78
4	PEL-10	17.20	19.11
5	PER-14	13.12	14.57
6	Control	0.00	-
S.Em. ±		0.41	-
C.D at 5%		1.15	-
C.V. (%)		4.58	-

Table 4: Quantification and quality of DNA samples obtained through NanoDrop spectrophotometer

Sr. No.	Name of isolates	260/280 nm	260/230 nm	DNA Concentration (ng/µl)
1	PET-19	1.78	1.45	315.8
2	PER-17	1.87	2.00	81.2
3	PER-2	1.84	1.54	115.5

Table 5: Morphological characterization of bacterial endophytes

Sr. No.	Endophytic bacterial isolate	Shape	Gram's reaction
1	PET-19	Rod	-ve
2	PER-17	Rod	+ve
3	PER-2	Rod	-ve
4	PEL-10	Rod	-ve
5	PER-14	Rod	-ve

Table 6: Biochemical characterization of endophytic bacterial isolates (Qualitative)

Sr. No.	Isolates	HCN production		Ammonia production	
		Reaction	Colour observed	Reaction	Colour observed
1.	PET-19	+++	Light brown	+++	Brown
2.	PER-17	-	Brown	+++	Brown
3.	PER-2	+++	Light brown	+++	Brown
4.	PEL-10	++	yellow	+++	Light Brown
5.	PER-14	-	yellow	+++	Brown
6.	Control	-	yellow	-	Greenish yellow

Note: Reaction categories: '-' negative, '++' moderate, '+++' strong

Table 7: Biochemical characterization of endophytic bacterial isolates (Quantitative)

Sr. No.	Isolates	Phosphate Solubilisation		Protease activity	
		Diameter of halo zone (mm)	Reaction observed	Diameter of halo zone (mm)	Reaction observed
1	PET-19	2.1	+	6.05	+
2	PER-17	3.9	+	5.20	+
3	PER-2	6.8	+	4.25	+
4	PEL-10	0.0	-	0.0	-
5	PER-14	3.5	+	0.0	-
6	Control	0.0	-	0.0	-

Note: + indicates the presence of a halo zone, - indicates the absence of the halo zone

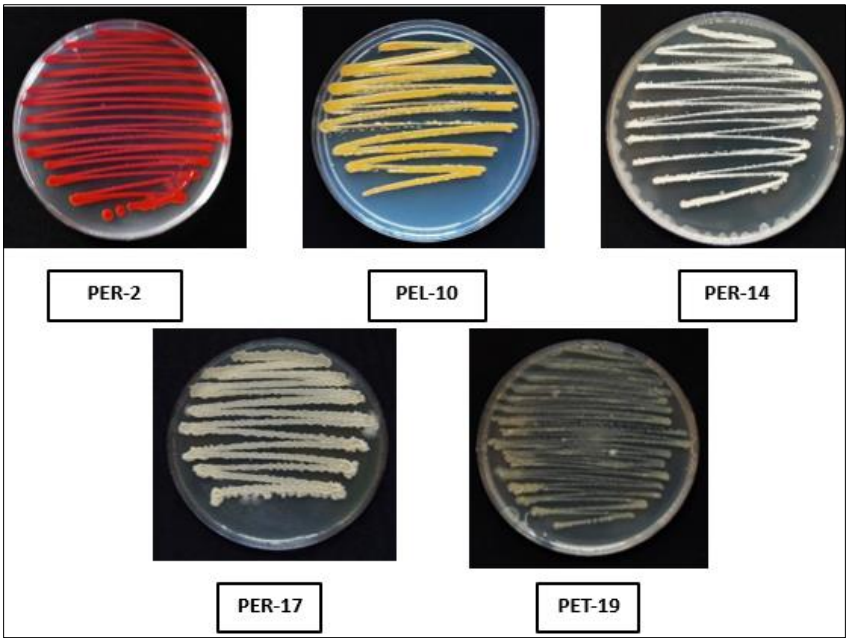

Plate 1: Pure cultures of bacterial endophytes isolated from different parts of potato after 3 days of incubation

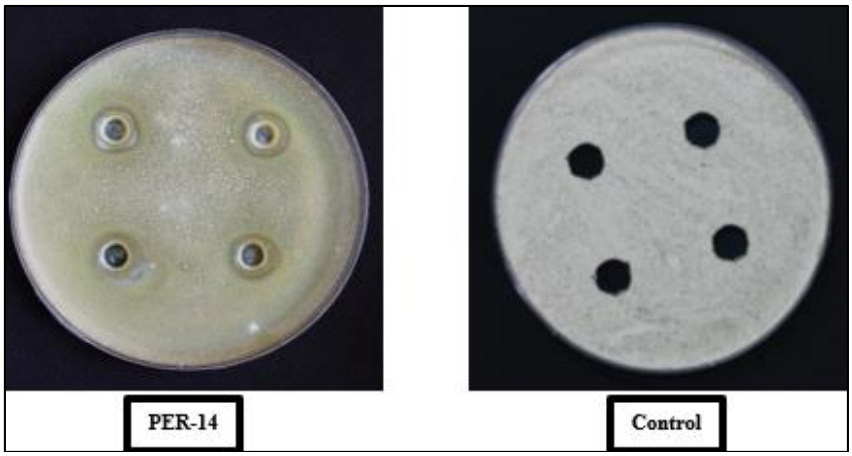

Plate 2: Effect of efficient endophytes against *S. scabies* by agar well diffusion method

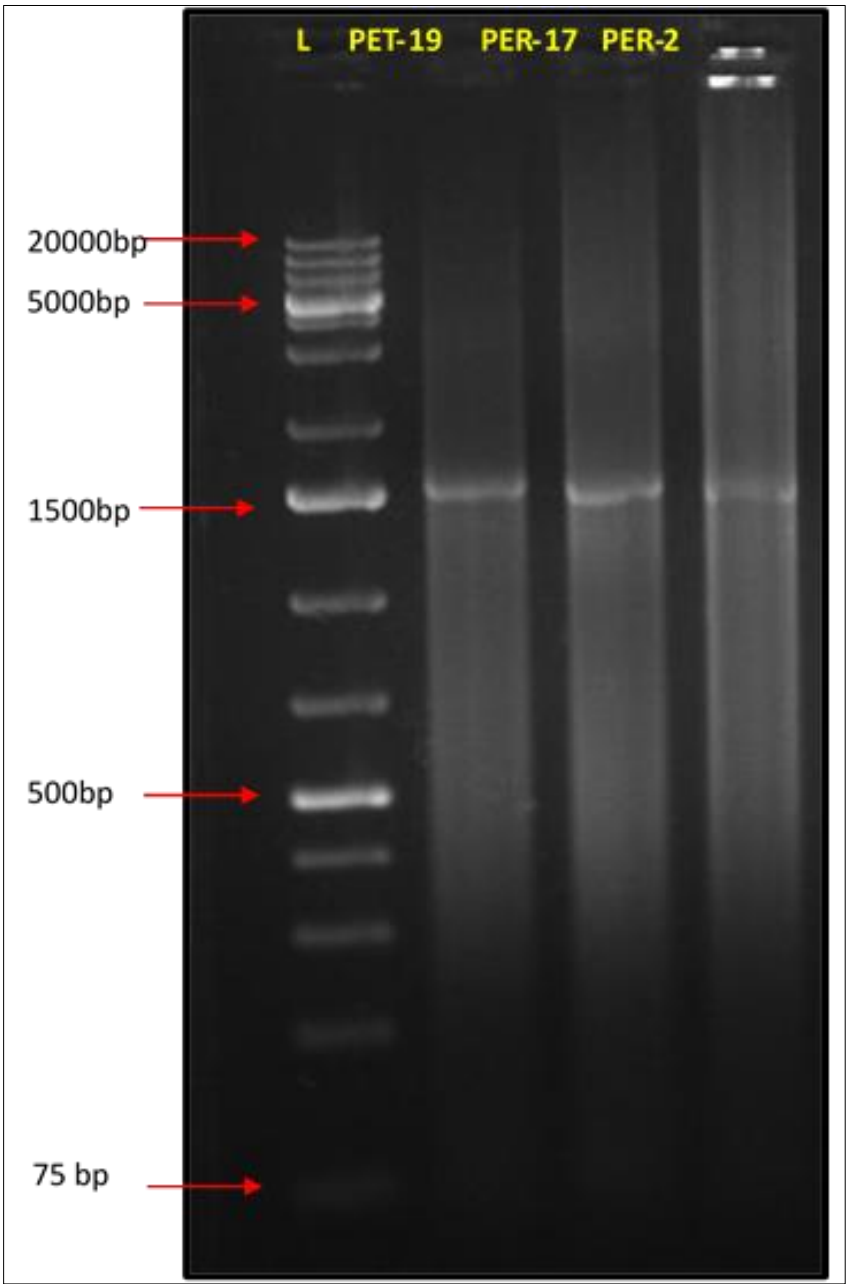

Plate 3: Amplified PCR product obtained on 1.8 % agarose gel electrophoresis
Note: L: Ladder, PET-19, PER-17 & PER-2: Endophytic DNA

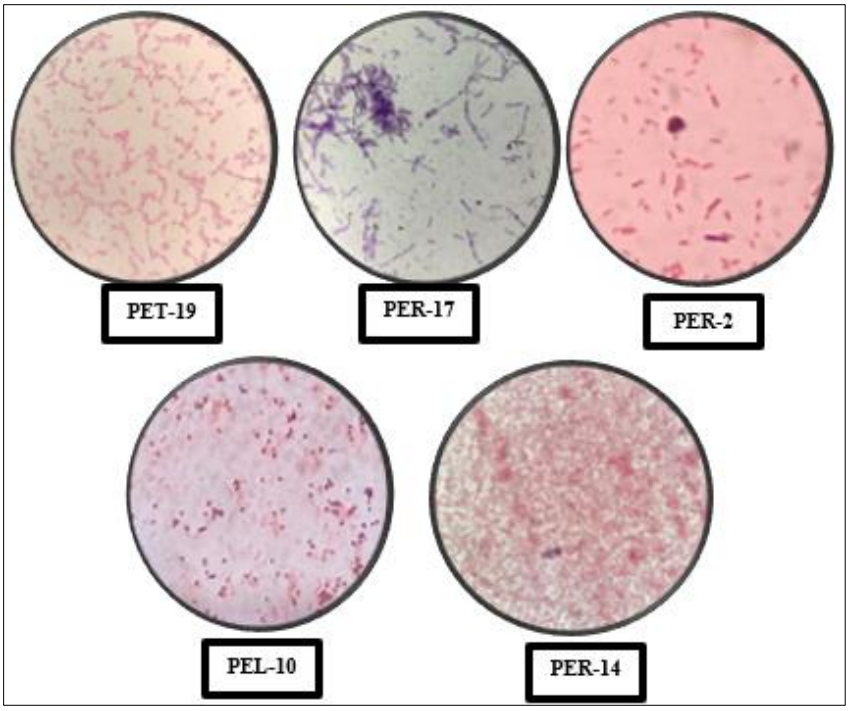

Plate 4: Gram staining of bacterial endophytes

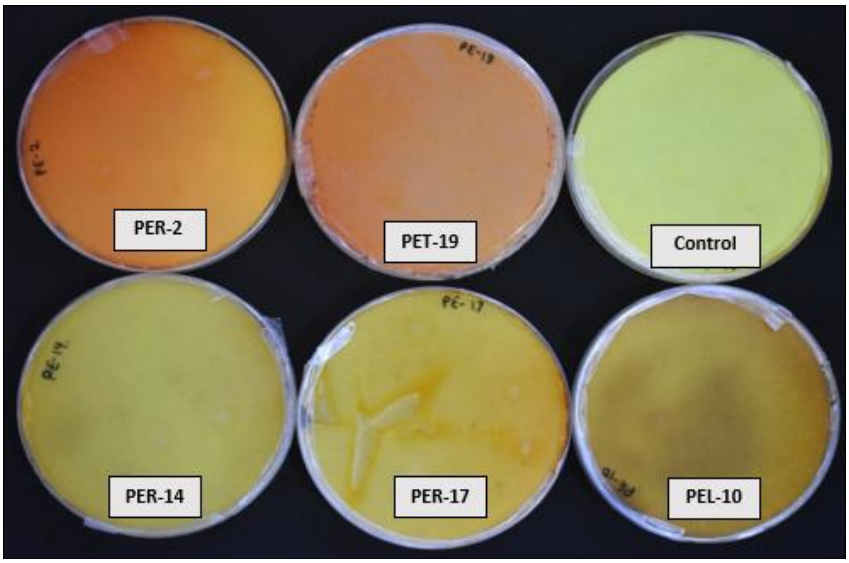

Plate 5: HCN production by bacterial endophytes

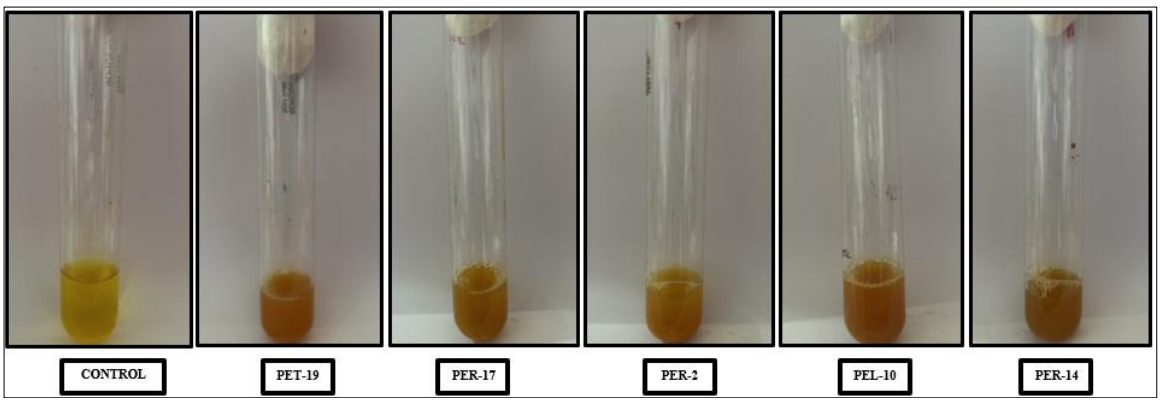

Plate 6: Ammonia production test of bacterial endophytes

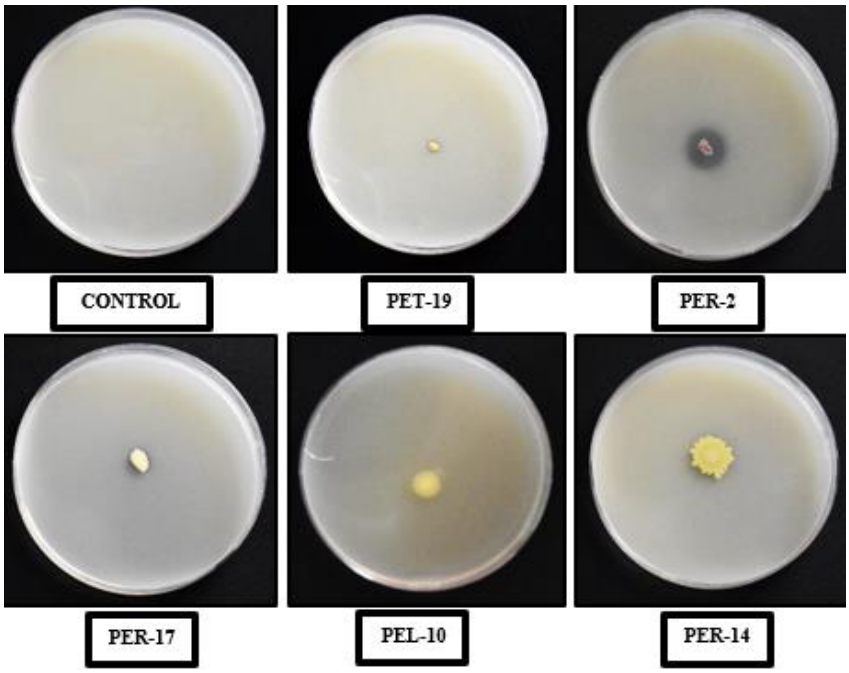

Plate 7: Phosphate solubilization by bacterial endophytes

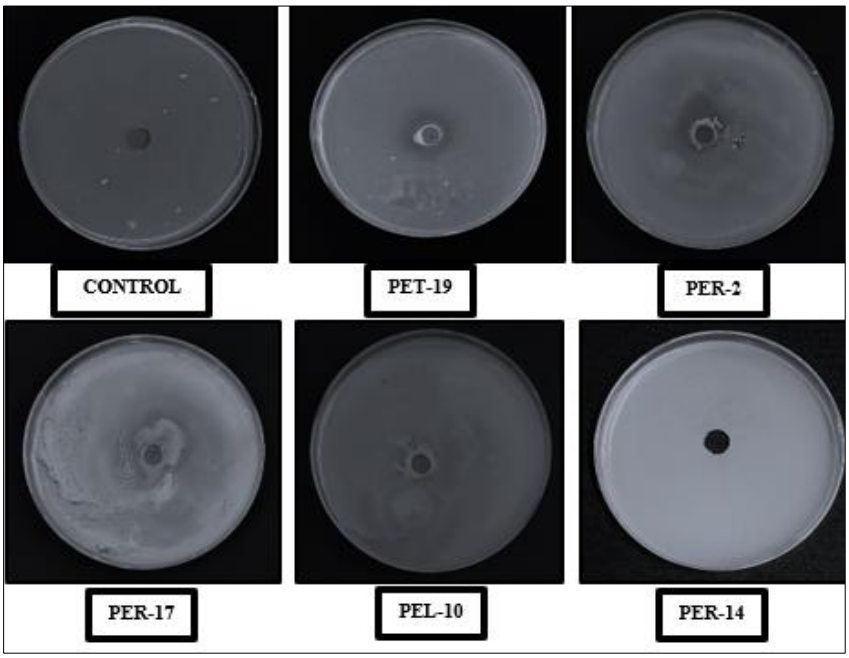

Plate 8: Protease production by bacterial endophytes

Conclusions

In this study, bacterial endophytes from healthy potato plants were isolated and evaluated for their efficacy against *Streptomyces scabies*. Out of 20 isolates, three demonstrated significant antagonistic activity. These isolates were identified as *Alcaligenes aquatilis*, *Bacillus altitudinis*, and *Serratia marcescens* through 16S rDNA sequencing. Further morphological and biochemical characterization revealed that all isolates, except for one, were Gram-negative, and they displayed various biochemical properties such as hydrogen cyanide production, ammonia production, phosphate solubilization, and protease activity.

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