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Title:	**Cataloguing Bacterial Endophytes of *Calotropis procera* against Potato Early Blight**

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Cataloguing Bacterial Endophytes of *Calotropis procera* against Potato Early Blight

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ABSTRACT

Background. Potato (*Solanum tuberosum* L.) is a vital food crop, worldwide. It is known for its high nutritious and economic significance, for being a cash and staple crop. However, in Pakistan, its production is seriously hampered by an early blight of potato caused by *Alternaria solani,* leading to excessive use of fungicides for its control. The current study was conducted to isolate and characterize the endophytic bacteria from *Calotropis procera* and to identify the secondary metabolites produced by endophytes with the highest antagonism against *A. solani*.

Methods. *C. procera* samples were collected from the fields of MNS University of Agri-culture Multan (MNSUAM). The endophytes were isolated from the leaves and roots of *C. procera,* followed by their morphological and biochemical characterization. The potato early blight samples were collected from the fields of MNSUAM and *A. solani* isolation was confirmed after their morphological identification. The 8 isolated endophytes were evaluated for their antagonism against potato early blight pathogen through dual culture assay. The best performing isolate was subjected to gas chromatography-mass spectrome-try (GC-MS) based secondary metabolites profiling.

Results. A total of 8 endophytic isolates were evaluated. Among the tested isolates, C[4] exhibited the highest antifungal activity (~70%), significantly inhibiting the growth of *A. solani.* Endophyte C4 produced potent antimicrobial metabolites that effectively suppressed pathogen growth. Based on GC-MS analysis, the major compounds identified were phenazine-1-carboxylic acid (antibiotic, anti-biofilm), oleic acid (membrane disruptor), and others.

Conclusion. The study demonstrated that endophytic bacteria isolated from *C. procera*, particularly isolate C4, possessed strong antagonistic activity against *A. solani*. The production of diverse antifungal metabolites highlights their potential as eco-friendly biocontrol agents. These findings support the evaluation of selected endophytes under greenhouse and field conditions for sustainable management of early blight in potato.

Keywords: *Alternaria solani*, antifungal metabolites, biocontrol agents, *Calotropis procera*, endophytes

Highlights

- Endophytes, particularly those isolated from medicinal plants like *C. procera*, showed promising potential for being effective biocontrol agents.

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- The isolate C4 showed maximum inhibition (~70%) and produced multiple antifungal metabolites, as revealed by GC-MS analysis.

- Endophytes can serve as a vital component of an eco-friendly biocontrol strategies for sustainable potato disease management.

GRAPHICAL ABSTRACT
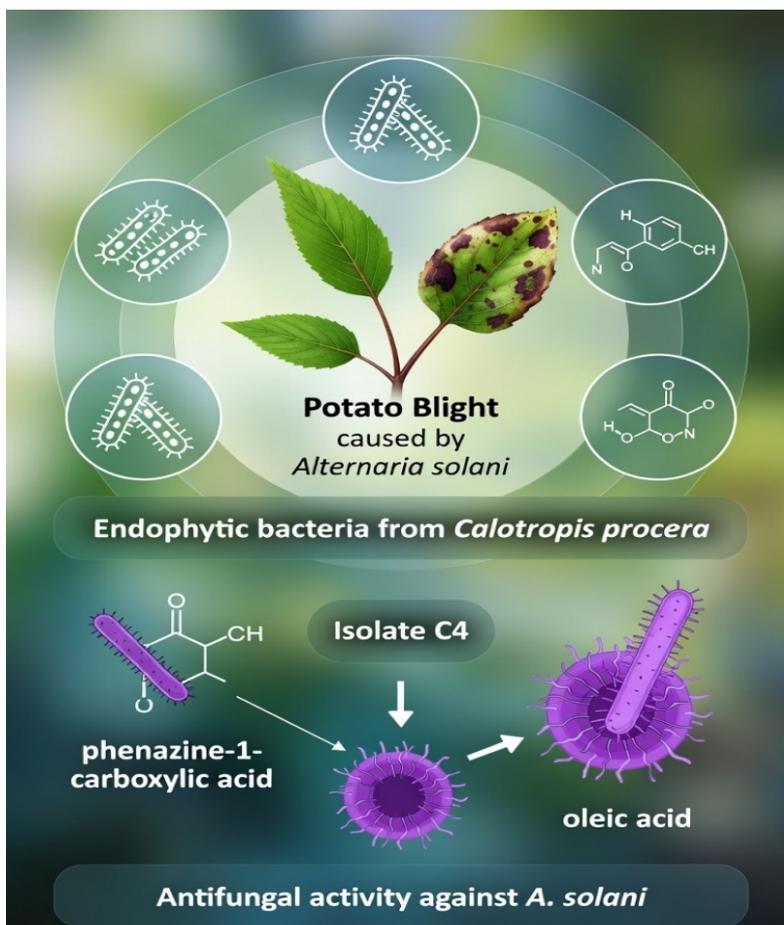
1. INTRODUCTION

Potato (*Solanum tuberosum* L) is one of the world's most important staple crops [1]. Archaeological and genetic studies confirm that Andean communities began cultivating wild potato species over 7,000 years ago [2]. In Pakistan, potato is grown in the months of January and February in the plains of the Baluchistan, Khyber Pakhtunkhwa, and Punjab [3]. The cultivated area of the potato crop has increased from 78.9 thousand hectares in 1995-96 to 172.8 thousand hectares in 2012-13 in the country, along with an increase in total production from 1,063.5 to 3,785.9 thousand tons [4]. Potatoes turn out to be an unexpectedly

powerful source of micronutrients, specifically vitamin C, vitamin B6, and potassium. One cooked potato (150g) can deliver 30-40% of the daily allowance of vitamin C, which is an important antioxidant needed by the body in the production of collagen, wound healing, and defending the body against disease [5]. There are more than 200 varieties of potato crop sold throughout the United States alone. These varieties fit into one of thirteen potato typess: Russet (Burbank), White Washed, Red/Pink Washed, Chat (Baby), Cocktail, Desiree, Yukon Gold, Purple, Medley, Fingerling, Kipfler, Japanese Sweet Potato, and Sweet Potato [6].

Potato production is destructively affected by abiotic factors including low temperature, frost, salinity, and nutrients deficiency, which influence plant growth [7]. Among the biotic stresses are fungi, bacteria, viruses, and nematodes. These diseases reduce potato production. Fungal diseases include early blight of potato (*Alternaria solani*), late blight of potato (*Phytophthora infestans*), and stem canker (*Rhizoctonia solani*) [8]. Bacterial diseases include bacterial wilt (*Ralstonia solanacearum*), ring rot (*Clavibacter michganensis*), and soft rot or blackleg (*Pectobacterium atrosepticum*). Apart from bacterial diseases, viral diseases such as potato virus Y, potato leaf roll virus, and potato cyst nematodes (*Globodera rostochiensis* and *Globodera pallida*) also impact potato production, globally [9]. Early blight also affects stem and tubers, which results in the yield loss of potato.

Early literature refers to yield damage of 5-50% [10]. When most of the yield has been produced, there emerges a difference between damage to vegetation and yield reduction, which is due to the upturn in disease extent at the end of the period [11]. To control the early blight of potato, different fungicides are used for disease (chlorothalonil, mancozeb, and copper fungicides) management. Systemic fungicides are absorbed in plant tissues and help to control against fungal infection, thus providing internal protection [12]. The results indicated that the repeated use of fungicides contaminates crops and the environment. The contamination of plants increases hazards regarding human and animal health [13]. In recent times, scientists have strived to develop alternate sources to control plant diseases, while reducing health risk and saving the environment. This is only possible through beneficial microbes because they have the ability to inhibit pathogen growth without harming the environment.

Plant endophytes show a promising potential for the management of plant diseases. They live within plant tissues without causing any visible symptoms of disease or harm to their host [14]. These microorganisms have proved to play numerous significant roles in enhancing plant growth, stress tolerance, and defense. Many endophytes have the capacity to synthesize bioactive molecules, which may enhance plant resistance against pathogens [15]. They produce a range of bioactive metabolites that can directly inhibit pathogen growth. These compounds include antibiotics, lipopeptides, volatile organic compounds (VOCs), and enzymes such as chitinases, glucanases, and proteases. Further, secondary metabolites have antifungal, antibacterial, and antiviral properties [16]. Certain fungal endophytes, such as those from the genus *Trichoderma*, produce chitinases, which degrade the cell walls of pathogenic fungi [17]. The *Pseudomonas* and *Bacillus* species produce phenazines and polyketides which incapacitate the growth of plant pathogens, such as *Xanthomonas* spp. and *Pseudomonas syringae* [18]. Bacterial endophytes induce systemic

resistance in plants by modulating jasmonic acid and ethylene dependent pathways which enables them to show resistance against pathogens [19]. In the case of *Calotropis procera*, a species of plant resistant to the conditions of a harsh environment, bacterial endophytes are instrumental in triggering resistance to diseases and pathogens. This study expounds the role of bacterial endophytes in the control of plant diseases, especially *C. procera,* which is reported to host several endophytes with proven antimicrobial potential [20, 21].

2. MATERIALS AND METHODS

2.1. Survey of Sahiwal Division and Sample Collection

A survey of potato fields was conducted to assess the incidence and severity of early blight of potato in Sahiwal division during the potato growing season. Samples with early blight symptoms (concentric rings) were randomly collected from 3 districts (Sahiwal, Pakpattan, and Okara). The collected leaves were carefully placed in sealed plastic bags and brought to the lab in MNS University of Agriculture, Multan [22].

2.2. Isolation and Morphological Identification of *Alternaria solani*

Potato dextrose agar was poured into sterilized petri plates, followed by surface sterilization of 3-5 mm infected leaves pieces. Surface sterilization was done with 70% ethanol solution for 2 minutes. Subsequently, petri plates were washed with sterilized water 3 times and dried on sterilized blotter paper. Leave pieces were placed on PDA containing petri plates and incubated at 28°C for 4 days. Fungal growth was observed and pure culture was obtained using single spore culture technique. The fungal colony was observed on the basis of its various characters, viz., color, growth, and pigmentation, followed by slide preparation to observe spore shape, cell wall thickness, septation, and color.

2.3. Isolation of Endophytes from *Calotropis procera*

The MNSUAM and nearby fields were surveyed for the collection of leaves and roots of *C. procera*. Then, samples were washed with running tap water to remove the dirt. The washed samples were allowed to dry before being cut into 5 mm sections using either scissors or a sterilized cutter. Each part of the sample, including stem, leaves, and roots, was surface sterilized by dipping it in a 5% sodium hypochlorite solution for 5 minutes. This was followed by immersion in 70% ethanol for 1 minute. Finally, the samples were rinsed thrice with sterile distilled water to eliminate any residual sodium hypochlorite and ethanol. Then, the samples were placed on sterilized blotter paper to absorb excess moisture. Sample pieces were placed on nutrient agar (NA) plates with the help of a sterilized needle in the laminar flow chamber. Petri plates were wrapped with a parafilm tape and placed in the incubator at 28°C. The plates were observed after 48-72 hours of incubation for bacterial growth.

2.4. Purification of Bacterial Endophytes

Bacterial culture was observed and sterilized loop was used to streak bacteria onto NA-containing plates employing the quadrant streak method. The plates were incubated for 24-48 hours at 28°C in order to obtain single colonies. These were later used to obtain single colony derived pure bacterial cultures.

2.5. Biochemical Characterization

2.5.1. KOH Test. A small amount of pure bacterial culture was picked with the help of a sterilized loop and placed on a

clean glass slide. One drop of KOH solution 3% was added to the smear on the slide and mixed gently to ensure that the bacteria remained properly exposed to KOH, followed by microscopic observation of the slide. The presence of mucus was observed to ascertain the gram-negative bacteria.

2.5.2. Gram Staining. A drop of distilled water was placed at the center of each clean slide. Then, a single colony of bacterial endophyte from the young culture was transferred onto the clean glass slide. A very thin film was created on each slide by spreading it evenly. The film was fixed by briefly passing the slide over a gentle flame two to three times. Following this, the slide was saturated with a crystal violet solution and allowed to sit for 30 seconds. Afterwards, it was rinsed with tap water. Next, it was immersed in iodine solution for 1 minute. Later, it was thoroughly washed with 95% alcohol for 10 seconds. The alcohol was drained off and the slide was rinsed with tap water. It was then treated with safranin for 1 minute. Finally, each slide was washed and rinsed once more with distilled water and allowed to air dry. The cellular morphology of inoculants was observed under a microscope.

2.5.3. Starch Hydrolysis Test. A sterilized inoculating loop was used to collect bacterial colonies from the poured isolates. A starch plate was streaked in a straight line across its width. Then, the streaked plates were incubated at 37°C for 48 hours. After the incubation period, 2-3 drops of 10% iodine solution were applied to the edges of the colonies and the results were documented after a gap of 10-15 minutes.

2.5.4. Catalase Test. A small drop of 3% hydrogen peroxide was placed on the sterilized slide. A single colony of bacteria was picked from the cultured plate with sterilized loop and placed on the drop. The

slide was observed for the formation of bubble foams that indicated a positive catalase test, confirming the presence of catalase enzyme and indicating the breakdown of hydrogen peroxide. In the absence of catalase enzyme, no bubbles were formed.

2.6. Morphological Identification of Bacterial Endophytes

After purification, a slide was prepared by placing a drop of fluid on its center with the help of micro pipette. One drop of distilled water was added to the center of the slide. A single colony was picked with a loop and gently mixed into the drop of distilled water using circular motion. A cover slip was placed on a thin smear on the glass slide and observed under a microscope to examine surface gloss, transparency, size, color, shape, and edge neatness.

2.7. Survey and Sample Collection

The survey of Sahiwal division in the Punjab province of Pakistan was conducted during the potato growing season. Samples with early blight symptoms (concentric rings) were randomly collected from its 3 districts (Sahiwal, Pakpattan, and Okara). The collected leaves were carefully placed in sealed plastic bags to prevent contamination and transported to the laboratory.

2.8. Disease Incidence

Disease incidence was recorded during field surveys conducted in the Sahiwal division. In each district, 200 plants at 3 different sites were randomly observed for the presence of disease symptoms. The number of diseased plants was counted and disease incidence was calculated using the following formula:

$$\text{Disease incidence (\%)} = \frac{\text{Number of infected plants}}{\text{Total number of obsreved plants}} \times 100$$

2.9. Disease Severity

Disease severity was assessed in the surveyed potato fields using a rating scale (Table 1).

Table 1. Disease Severity Rating Scale

Grade	Severity Percentage	Symptoms on Leaves	Description
0	0%	No visible symptoms	Healthy plant
1	0-5%	1-2 small spots on a few leaves	Very slight infection
2	6-10%	Few scattered lesion, lower leaves affected	Slight infection
3	11-25%	Noticeable lesion on several leaves	Moderate infection
4	26-50%	Many leaves affected	High infection
5	51-75%	More than half of plant infected	Severe infection
6	76-100%	Whole affected or dead	Very sever

At each selected site, 200 plants were randomly observed and the severity of infection in each plant was scored according to the scale. The collected data was then used to calculate the percent disease index using the following formula:

$$\text{Disease severity (\%)} = \frac{\text{Sum of (Rtaing} \times \text{Leaves)}}{\text{Total Leaves} \times \text{Max Rating}} \times 100$$

2.10. *In vitro* Screening of Various Endophytic Antagonistic Bacteria against *A. solani* by using Line Streaking Method

Antagonistic isolates were grown individually on the PDA medium under optimal growth conditions to evaluate their efficacy against fungal pathogen using the dual culture technique. The mycelium of fungus were cut out aseptically from the 3-day old culture with the help of a sterile cork borer and placed near one edge of the petri plate, while maintaining a uniform distance of 3 cm from the corner. The endophytic bacterial isolate culture was streaked on the center of the petri plate. The plates were inoculated only with the fungal pathogen, without bacterial antagonistic served as the control treatment. All plates were incubated at 28°C for a specified period under aseptic conditions. The interaction between the bacterial isolates and fungal pathogen was observed at 24-hour intervals for 7 days. The degree of inhibition was recorded on the basis of reduced growth of fungal mycelium in the presence of endophytic bacteria. The percentage inhibition of fungal growth was calculated using the following formula:

$$\text{Inhibition} = \frac{(C - T)}{C} \times 100\%$$

C = Colony diameter in control, T = Colony diameter in treatment

2.11. Sample Preparation for Metabolite Extraction

Using a sterile loop, a single colony of bacteria was taken out of the bacterial culture and inoculated into a flask containing 500 ml LB media. The flask was incubated at 28°C in a shaking incubator at the speed of 180 rpm. The centrifugation of bacterial cells in suspension was done at 10000g after an incubation period of 10 minutes. A pellet was attained by centrifugation at 10000g and 4°C for 20 minutes, followed by the addition of chloroform/methanol

(2:1) mixture. The solvent was evaporated using a vacuum rotary evaporator at 37°C. The resulting precipitation was weighed and solubilized in methanol to achieve a concentration of 10 mg ml^{-1}. The prepared sample was analysed using mass spectrometry on Q Executive Plus (Thermo Fisher Scientific, U.S.A.) equipped with an electrospray ionization (ESI) ion resource and Xcalibur workstation. The experiment was carried out at 250°C through direct injection in positive ionization mode capillary with a spray voltage set at 2,300 (V).

3. RESULTS

3.1. Disease Incidence and Severity in Sahiwal Division

The survey was conducted during the potato growing season in Punjab, Pakistan. Samples with early blight symptoms (concentric rings; Figure 1) were randomly collected from the three districts of Sahiwal division (Sahiwal, Pakpattan and Okara). The maximum disease incidence, that is, 18% was observed in Sahiwal, followed by 11.36% and 7.69% in Pakpattan and Okara, respectively (Figure 2).

Figure 1. Small, Dark, Circular to Angular Lesions on Leaves, with Concentric Rings

3.2. Morphological Identification of *A. solani*

After isolating pure culture, the pathogen was identified on the basis of colony characters, viz., color, growth, and pigmentation.
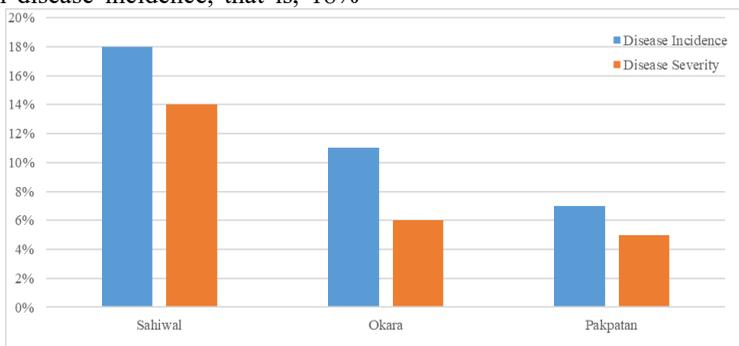

Figure 2. Disease Incidence and Severity in Sahiwal Division

Figure 3. Pure Colony of *A. solani*
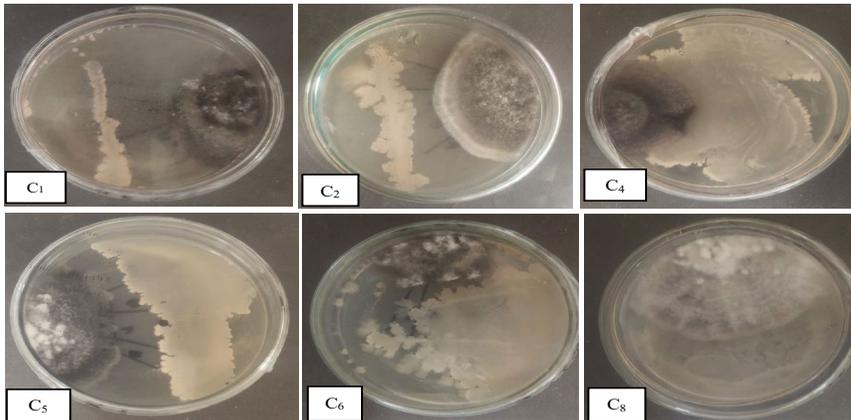

Figure 4. *A. solani* Spores

Figure 5. Antagonistic Effects of Different Endophytic Bacteria

3.3 *In vitro* Efficacy of Different Endophytic Bacteria against *A. solani*

The isolates of endophytic bacteria (C1-C8) were evaluated against *A. solani* for their antifungal activity. Dual culture test was conducted in the diagnostic lab of MNSUAM. Among all isolates, C4 exhibited the highest inhibitory effect with mycelium growth inhibition at 70%. This was followed by C6 which showed moderate inhibition levels of about 55%. Based on the results, it was determined that the C4-C6 cloud inhibited the colony growth of *A. solani*o (Figure 5).

Figure 6. Control

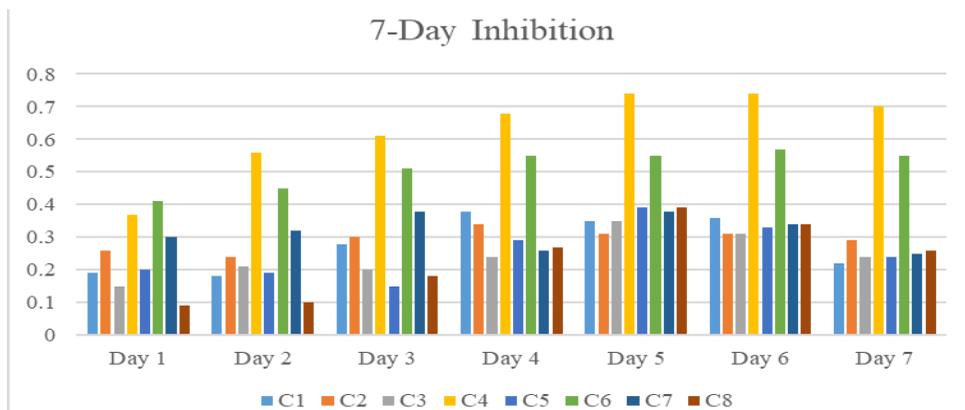### 3.4. *In vitro* Efficacy of Endophytic Bacteria against *A. solani*

Figure 7. Efficacy of Different Bacterial Endophytes Treatments against *A. solani*

Table 2. Biochemical Characterization

Bacteria	KOH Test			Gram Staining Test		Starch Hydrolysis Test		Catalase Test	
	Observation	Sting formation	Results*	Microscopic Observation	Gram reaction*	Zone of clearing After Iodine	Results*	Formation of bubbles	Results*
C1	Thick, sticky thread	Yes	-	Purple	-	Clear halo observed	-	Absence	-
C2	No viscosity, no string	No	+	Pink	+	No clear zone	+	Present	+
C3	Thick, sticky thread	Yes	-	Purple	+	Slight clearing	+	Absence	+
C4	Thick, sticky thread	Yes	-	Pink	-	No clear zone	+	Absence	+
C5	Thick, sticky thread	Yes	+	Purple	-	No clear zone	+	Absence	-
C6	Thick, sticky thread	Yes	-	Purple	+	Clear halo observed	-	Present	+
C7	No viscosity, no string	No	+	Pink	-	Clear halo observed	+	Present	+
C8	No viscosity, no string	No	+	Purple	-	Clear halo observed	+	Absence	-

*positive (+) /Negative (-)3.5.1 Morphological Identification of Endophytic Bacteria

Table 3. Morphological Identification of Endophytic Bacteria

Bacteria	Tissue Source	Color	Shape	Margi	Cell Shape
C1	Root	Cream White	Round	Entire	Rod
C2	Root	Yellowish	Circular	Wavy	Cocci
C3	Leaf	Off-white	irregular	Undulate	Rod
C4	Root	Pale Yellow	Round	Entire	Cocci
C5	Leaf	Yellowish	Circular	Wavy	Rod
C6	Leaf	Off-white	Irregular	Convex	Rod
C7	Root	Cream White	Circular	Raised	Cocci
C8	Leaf	Off-white	Round	Convex	Rod

3.5. Biocontrol Potential of Endophytes against *A. solani* Inhibition Zones and GC-MS Findings

Based on the inhibition zone data (mm), Treatment 2 demonstrated the highest biocontrol potential against the early blight pathogen (*A. solani*), particularly on Day 5 (3.13 mm), which was significantly higher than other treatments (except the control). This suggests that endophyte C4 produced potent antimicrobial metabolites that effectively suppressed pathogen growth. Based on GC-MS analysis, the major compounds identified were phenazine-1-carboxylic acid (antibiotic, anti-biofilm), oleic acid (membrane disruptor), cyclo (L-Pro-L-Tyr) (antimicrobial diketopiperazine), and 9-Octadecenamide (fungal cell wall inhibitor).

4. DISCUSSION

The early blight of potato caused by *A. solani* significantly limits potato production and requires frequent sprays of its fungicide. In the current study, endophytes were isolated from *Calotropis procera,* which showed significant antagonistic activity against early blight pathogen, reflecting the notion of medicinal plants as hosts of endophytes with the bioactive potential to suppress the fungi [23]. The results showed that endophytic bacteria C4 demonstrated the highest mycelial inhibition (~70%), underlining its effective antagonistic potential. The antagonistic effects of bacterial metabolites against plant pathogens showed the promising potential of endophytes as biocontrol agents [24, 25, 26], particularly those isolated from *Calotropis procera* [27]. GC-MS analysis of C4 revealed phenazin-1-carboxylic acid (PCA), a redox-active compound with antibiotic potential and widely known to induce oxidative stress and disrupt fungal respiration. The documented endophytes with biocontrol potential are known to produce PCA [28, 29]. In addition to PCA, some fatty acids like oleic acid can severely damage fungal cell membrane, leading to limited fungal growth and inhibition. The biological importance of PCA, oleic acid, cyclo (L-Pro-L-Tyr), and 9-Octadecenamide lies in their potential to prevent bacterial biofilm production, disruption of cell membrane, and fungal cell wall receptors inhibition for pathogenic activity. The multifaceted inhibiting mechanism employed by metabolites suppress fungal growth and reduce the risk of resistance development [30]. The results from *in vitro* assays underline the need for greenhouse and field evaluation before integrating these endophytes in early blight management regime, thus minimizing the fungicide use.

4.1. Conclusion

This study examines the potential of endophytic bacteria against *Alternaria solani,* causal agent of early blight of potato. In this regard, C4 and C6 demonstrated significant antifungal activity. By producing antifungal metabolites such as PCA and oleic acid, the above compounds inhibited fungal growth and spores production. The findings highlight the potential of endophytic bacteria for sustainable disease management strategies as an eco-friendly alternative to chemical fungicides. Further field-based evaluation and formulation development are recommended to incorporate the results into practical agricultural applications.

Author Contribution

Abdul Hadi: investigation, methodology, writing - original draft. **Hasan Riaz:** conceptualization, methodology, formal analysis, writing - review & editing. **Muhammad Shahzad Zafar:** conceptualization, writing - review & editing.

Conflict of Interest

The authors of the manuscript have no financial or non-financial conflict of interest in the subject matter or materials discussed in the manuscript

Data Availability Statement

Data supporting the findings of this study will be made available by the corresponding author upon request.

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Generative AI Disclosure Statement

The authors did not use any type of generative AI software for this research.

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