

Antimicrobial and plant growth-promoting activities of bacterial endophytes isolated from *Calotropis procera* (Ait.) W.T. Aiton

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Abstract: Bacterial endophytes are beneficial to their hosts as they can fix nitrogen in the soil and make it available to the host. Endophytic bacteria also secrete plant growth-promoting hormones to support their host plants under normal as well as stress conditions. The current study aimed to isolate endophytic bacteria from different parts of *Calotropis procera*, i.e., roots, stem and leaves of *Calotropis procera* (Ait.) W.T. Aiton. Plants were collected from the Lundkhwar, district Mardan. A total of 12 bacterial strains, i.e., six from roots, three from the stem and three from the leaves were isolated. The strains were screened for their growth-promoting activity in rice plants because rice shows a quick and easy response to the bioactive compounds present in the culture filtrate (CF) of the potent endophytic strains. The rice plants were cultivated in pots containing 30 mL of 0.8% w/v water-agar medium. The pots were placed in a growth chamber, operated at $28 \pm 0.3^\circ\text{C}$ for 14 h (day); and $25 \pm 0.3^\circ\text{C}$ for 10 h (night), at 70% relative-humidity. Among the isolated strains, R1, S1, S3, L1, R5 and R6 showed visible growth promotion in rice plants. The biochemical analysis revealed that the strains were able to produce indole acetic acid (IAA) and flavonoids in higher quantities. Moreover, the strains also produced bioactive compounds that inhibited the growth of *Escherichia coli* and *Aspergillus flavus* using the well diffusion method. From the results, it was concluded that these strains can secrete potent compounds that can promote the host plant growth and inhibit the growth of pathogenic microorganisms and, therefore, can be used as bio-fertilizer and bio-control agents.

Introduction

Calotropis procera (Ait.) W.T. Aiton belongs to the family Apocynaceae. It is an erect and branched shrub containing milky latex, which is widely used as a medicinal plant in the Indian sub-continent. It has ethnobotanical importance in traditional medicines and is used for the treatment of various diseases (Pattnaik *et al.*, 2017). Besides the ethnobotanical importance, *C. procera* acts as a reservoir for potent endophytes (Nagda *et al.*, 2017; Rani *et al.*, 2017).

Endophytes are the organisms that establish a mutual yet beneficial relationship with their host plants during long-term

evolutionary processes (Ali *et al.*, 2019; Bilal *et al.*, 2018; Gul Jan *et al.*, 2019). Endophytic microbes have the capability to produce a variety of bioactive compounds (Bibi *et al.*, 2018; Hamayun *et al.*, 2017; Ikram *et al.*, 2018). Endophytic microbes live within the plant tissues without harming the host plant (Ismail *et al.*, 2020a; Ismail *et al.*, 2019; Ismail *et al.*, 2018). A variety of endophytic bacterial species are found in the tissues of plants such as *Azospirillum*, *Pseudomonas* and *Bacillus* that can be extracted from internal plant parts or from surface-sterilized plant tissues (Phetcharat and Duangpaeng, 2012). The tissues of most plants contain endophytes that secrete secondary metabolites to regulate the plant metabolism, even under stress conditions (Ismail *et al.*, 2020b; Ismail *et al.*, 2020c; Jan *et al.*, 2019). Endophytes can act as biofertilizers as they can promote the growth of the host species under normal as

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well as stress conditions (Kang *et al.*, 2019; Khushdil *et al.*, 2019; Mehmood *et al.*, 2019). Endophytes enhance the growth of the host plant by secreting plant hormones, regulating the stomatal opening, enhancing nutrient absorption, and converting the heavy metals from unstable to stable form in the agricultural soil (Muhammad *et al.*, 2019; Nusrat *et al.*, 2019; Qadir *et al.*, 2020). Endophytic bacteria promote the growth of host plants by producing IAA, GA, ACC deaminase, exhibiting phosphate solubilization and siderophore activity, and biologically fix nitrogen fixation (Hamayun *et al.*, 2017; Ikram *et al.*, 2018). Endophytic bacteria are indicated to have an excellent ability to increase plant growth ratios in various ways as they secrete different forms of secondary metabolites in the tissues of that particular plant (Etesami *et al.*, 2014). In recent years, it has been noticed that endophytic bacteria protected the host plants against the nematodes (Mhatre *et al.*, 2019; Su *et al.*, 2017). Moreover, it is observed that bacterial endophytes have an advantageous role in protecting host plants by increasing the phytoremediation process in heavy metals contaminated soils (Zam *et al.*, 2016). Therefore, for the promotion of plant growth, beneficial bacteria may be introduced to the soil to get maximum benefits in terms of high yield. To achieve this aim, endophytic bacteria were isolated from different parts, i.e., roots, stems, leaves of *C. procera* and screened for plant growth-promoting and antimicrobial activities.

Materials and Methods

Plant materials

Fresh samples of *C. procera* were collected from village Lund Khwar (71°59' E, 34°23' N with an altitude of 371 m) in district Mardan, Pakistan. The plant samples were collected in polythene bags and safely transferred to the plant-microbe interactions laboratory for further investigation.

Isolation and purification of bacterial endophytes

Plant samples were washed in running tap water to remove dust and debris. The washed plant samples were then cut into parts, i.e., stem, leaves and roots, with the help of sterilized scalpels. The separated plant parts were surface-sterilized by dipping them in 5% sodium hypochlorite solution for 5 min, followed by 70% ethanol for 1 min, and finally rinsed three times in sterile distilled water to remove any traces of sodium hypochlorite and ethanol. From each sterilized plant part, 1 g of tissue was aseptically macerated in 9 mL sterile saline solution using pestle and mortar. About 1 mL of macerated sample was taken and spread on tryptic soy agar and/or nutrient agar plates. The plates were transferred to the incubator, which was then incubated at 37°C for 72 h. The plates were observed for the appearance of bacterial colonies on a daily basis. The plates showed the presence of different bacterial colonies was transferred to the potato agar plates for further purification. Pure colonies were identified by observing the size, color, shape, and growth pattern (Barillot *et al.*, 2013; García-Salamanca *et al.*, 2013).

Indole-3-acetic acid and flavonoids determination

IAA production was examined by a well-established method (Loaces *et al.*, 2011). Briefly, each bacterial suspension

(1×10^8 CFU/mL) was inoculated in 10 mL LB broth containing L-tryptophan (100 g/mL) in a 50 mL falcon tube. The broth was incubated at 28°C and 200 rpm for 72 h. Bacterial cells were precipitated by centrifugation (8,000 rpm for 15 min), and the collected supernatant was incubated at room temperature in the dark for 30 min. Pure IAA (Sigma, USA) was used as a standard. The IAA concentration in the culture supernatant was measured at 530 nm with Salkowski's reagent (12 g/L FeCl₃ in 7.9 M H₂SO₄). Each experiment was repeated three times.

Total flavonoids were estimated by using the aluminum chloride colorimetric method (Saravanan and Parimelazhagan, 2014). In a test tube, 4.3 mL of methanol (80%) was added to 0.5 mL of each culture filtrate. Then, 0.1 mL of aluminum chloride (10%) and 0.1 mL of potassium acetate (10%) was added to the tubes. The samples were incubated for 30 min at room temperature. After incubation, the samples were shaken, and the absorbance was recorded at 510 nm.

Screening for the potent strains

To assess the growth activity of the bacterial isolates, rice was used as a test plant species. Rice was selected because of the quick response to the plant growth promoters and inhibitors. Initially, seeds of rice were surface-sterilized, as mentioned earlier. The clean seeds were spread in a plate containing sterilized distilled H₂O for germination. Germinated seedlings of uniform size (5 per pot) were transplanted in the pots containing 30 mL of 0.8% w/v water-agar medium. The pots were placed in a growth chamber, operated at 28 ± 0.3°C for 14 h (day) and 25 ± 0.3°C for 10 h (night), at 70% relative-humidity. When the tested rice cultivars reached a two-leaved stage, a 10 µL of lyophilized bacterial filtrate suspension was applied to the tip of the apical meristem. The growth attributes of both rice cultivars were analyzed after 10 days of treatment. The experiment was carried in triplicate.

Estimation of rice growth parameters

Root and shoot length was measured manually with the help of a scale. Plant fresh weight was measured directly using an analytical balance, while plant dry weight was estimated after drying the samples in an oven for 48 h at 70°C.

Collection of pathogenic microbes

Escherichia coli and *Aspergillus flavus* were collected from the laboratory of the Centre of Biotechnology and Microbiology, University of Peshawar.

Antibacterial assay

The antibacterial activity of the extracts was evaluated against the selected pathogenic bacterial strains, i.e., *E. coli* by agar well-diffusion technique (Zerroug *et al.*, 2018). Each bacterial pathogen (1×10^{-7} CFU/mL) was inoculated into Muller-Hinton agar plates. In each plate, three wells were made using a sterile cork borer. Wells were filled with 100 µL of the endophytic bacterial extract and distilled water as a blank. About 100 µL of standard ampicillin (30 µg/mL) was used as a positive control. Extracts were allowed to diffuse through agar media at room temperature for 2 h, and then the plates were incubated for 24 h at 37°C. The

diameters of inhibition zones were recorded. The experiment was repeated three times.

Antifungal activity

Potato dextrose agar media were used to test the potency of isolated bacterial endophytes from *C. procerca* against the pathogenic *A. flavus* strain according to a standard protocol (Dellavalle *et al.*, 2011).

Statistical analysis

All the experiments were performed in triplicate. ANOVA (one-way analysis of variance) was used for the analysis of data and means were compared by Duncan multiple range test at $p < 0.05$, using SPSS for Windows 16.0 (SPSS Inc., Chicago, IL, USA). Graphs were constructed by using Graphpad Prism 6.0.

Results

The study was conducted at Plant-microbes interaction laboratory, Department of Botany, Abdul Wali Khan, University, Mardan. *Calotropis procerca* was collected from village Lund Khwar, district Mardan to isolate potent endophytic bacterial strains from different parts of the plants, i.e., leaves, stems and roots. A total of 12 bacterial strains was isolated from various parts of *C. procerca*, i.e., 6 bacterial strains (R1, R2, R3, R4, R5 and R6) were isolated from the roots, 3 strains (L1, L2, L3) from the leaves, and 3 strains (S1, S2 and S3) from the stem.

Plant growth parameters

Among the 12 isolated bacterial strains, the R1 promoted shoot and root length and was considered a potent strain as it increased both shoot and root length of rice plants, while L2 inhibited both shoot and root lengths. The extract of S3 also promoted root and shoot length (Fig. 1).

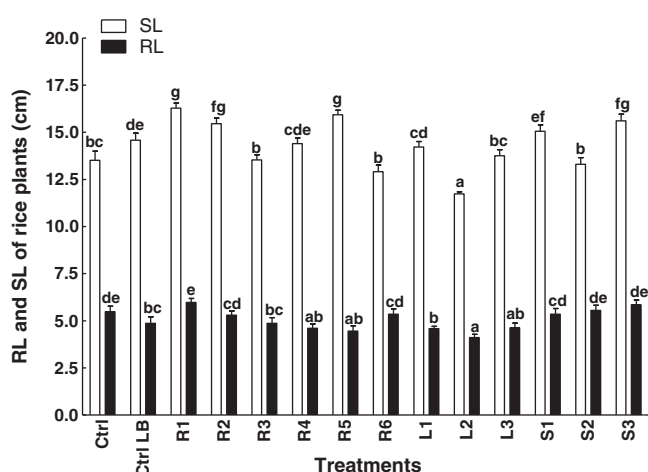


FIGURE 1. Effect of bacterial endophytes isolated from the various parts of the *C. procerca* on shoot and root length of rice plants. Data are the mean of three replicates with standard error bars. Means that are followed by different letters are significantly different ($p = 0.05$) from their respective bars. Ctrl: control with distilled water; Ctrl LB: control with the media; R1, R2, R3, R4, R5, R6: endophytes isolated from the roots of the *C. procerca*; L1, L2, L3: endophytes isolated from the leaves of the *C. procerca*; S1, S2, S3: endophytes isolated from the stem of the *C. procerca*.

A total of twelve bacterial extracts was applied to the rice plants. An increase in shoot fresh weight was observed in plants treated with S3 strain isolated from the stem of *C. procerca*. However, a more pronounced effect in terms of weight was recorded in plants treated with the extract from the R1 strain, which was isolated from the root (Fig. 2). Moreover, the other isolated bacterial strains from *C. procerca* did not show any significant changes in root and shoot weight as compared to the control plants (Fig. 2).

The effect of different endophytic bacterial extracts on the dry weight of the rice plant was measured (Fig. 3). Extracts were applied from the embryonic stage to their two-leaf stage. As compared to the controls, the extract of L1 increased the shoots dry weight of the rice plants. However, in the case of root dry weight, the R3 significantly enhanced the root dry weight of rice plants (Fig. 3).

Indole-3-acetic acid and flavonoid contents

IAA contents of the isolated bacterial strains from various parts of *C. procerca* were investigated (Fig. 4). The bacterial isolates from roots consisted of the strongest and weakest strains in terms of IAA production. R5 had the highest IAA producing ability, whereas the lowest contents were produced by the isolate R2 (Fig. 4). All the tested strains were capable of producing flavonoids (Fig. 5). The highest flavonoid contents were observed in the R6 treatment, while the lowest flavonoid contents were detected in S3 (Fig. 5).

Antibacterial and antifungal activity

The antibacterial activity was tested by using the isolated strains against *E. coli* (Tab. 1). The well diffusion method was utilized, and the results were compared with the positive and negative controls. The highest value for inhibition was shown by L1 (8.6 ± 0.7), which was isolated

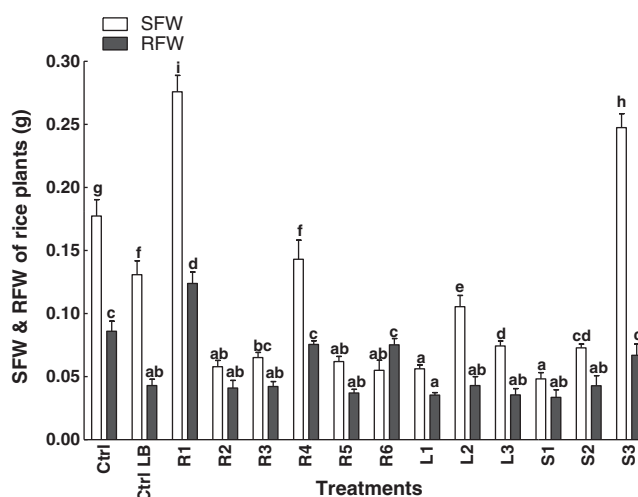


FIGURE 2. Effect of bacterial endophytes isolated from the various parts of the *C. procerca* on root and shoot fresh weight of rice plant. Data are the mean of three replicates with standard error bars. Means that are followed by different letters are significantly different ($p = 0.05$) from their respective bars. Ctrl: control with distilled water; Ctrl LB: control with the media; R1, R2, R3, R4, R5, R6: endophytes isolated from the roots of the *C. procerca*; L1, L2, L3: endophytes isolated from the leaves of the *C. procerca*; S1, S2, S3: endophytes isolated from the stem of the *C. procerca*.

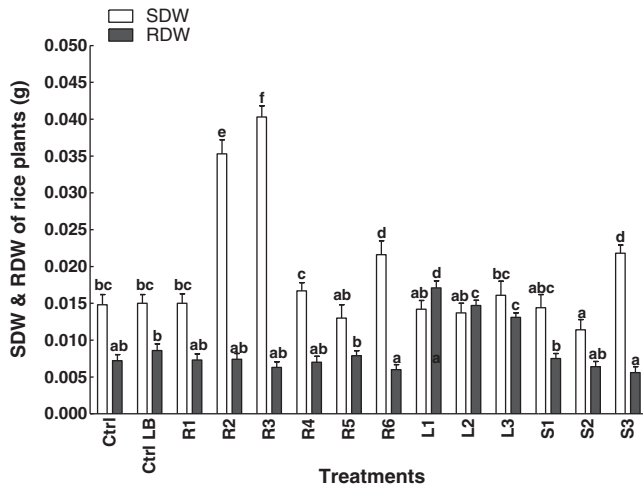


FIGURE 3. Effect of bacterial endophytes isolated from the various parts of the *C. procera* on root and shoot dry weight of rice plant. Data are the mean of three replicates with standard error bars. Means that are followed by different letters are significantly different ($p = 0.05$) from their respective bars. Ctrl: control with distilled water; Ctrl LB: control with the media; R1, R2, R3, R4, R5, R6: endophytes isolated from the roots of the *C. procera*; L1, L2, L3: endophytes isolated from the leaves of the *C. procera*; S1, S2, S3: endophytes isolated from the stem of the *C. procera*.

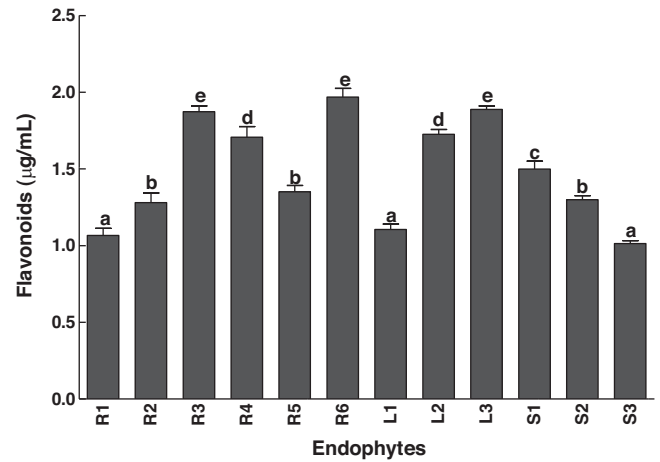


FIGURE 5. Secretion of total flavonoids by bacterial endophytes isolated from the various parts of the *C. procera*. Data are the mean of three replicates with standard error bars. Means that are followed by different letters are significantly different ($P=0.05$) from their respective bars. R1, R2, R3, R4, R5, R6: endophytes isolated from the roots of the *C. procera*; L1, L2, L3: endophytes isolated from the leaves of the *C. procera*; S1, S2, S3: endophytes isolated from the stem of the *C. procera*.

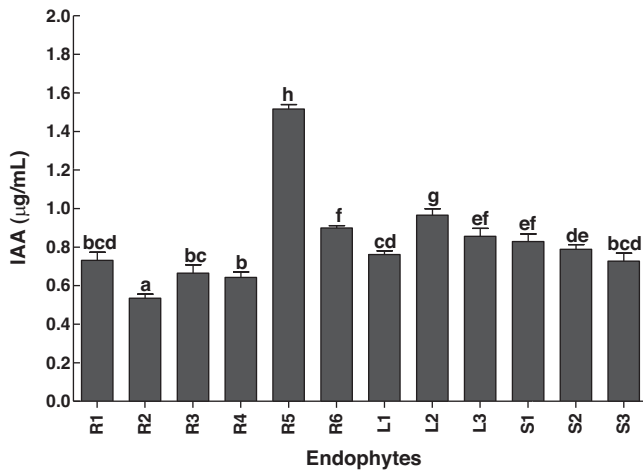


FIGURE 4. Production of IAA by bacterial endophytes isolated from the various parts of the *C. procera*. Data are the mean of three replicates with standard error bars. Means that are followed by different letters are significantly different ($p = 0.05$) from their respective bars. R1, R2, R3, R4, R5, R6: endophytes isolated from the roots of the *C. procera*; L1, L2, L3: endophytes isolated from the leaves of the *C. procera*; S1, S2, S3: endophytes isolated from the stem of the *C. procera*.

from the leaves. The lowest antibacterial activity was shown by L3 (2.2 ± 0.4), while the effect of L2 (4.3 ± 0.8) was moderate. The isolated endophytes from roots of *C. procerashowed* variable antibacterial activities, R4 (7.4 ± 0.1) led the antibacterial activity, followed by R3 (5.8 ± 0.7), R1 (5.2 ± 0.6), R6 (4.5 ± 0.4), R5 (4.4 ± 1.1), respectively. The R2 strain isolated from the *C. proceraroots* showed minimum antibacterial activity (4.0 ± 0.5). The isolated strains from the stem of *Calotropis procera* also showed variable antibacterial activities (Tab. 1). The strain S2 (6.7 ± 0.8) showed the highest antibacterial activity, followed by S3

TABLE 1

Antibacterial and antifungal properties of bacterial strains isolated from different parts of *C. procera*

Treatments	Antibacterial (zone of inhibition in mm)	Antifungal (zone of inhibition in mm)
C. L. B	0.5 ± 0.08	0.00 ± 0.0
Ampicillin	4.2 ± 0.2	3.2 ± 0.6
R1	5.2 ± 0.6	4.1 ± 0.6
R2	4.0 ± 0.5	3.6 ± 0.4
R3	5.8 ± 0.7	3.0 ± 1.0
R4	7.4 ± 0.1	2.8 ± 0.5
R5	4.4 ± 0.1	2.1 ± 0.1
R6	4.5 ± 2.4	1.8 ± 0.5
L1	8.6 ± 0.7	2.2 ± 0.4
L2	4.3 ± 0.8	2.2 ± 0.7
L3	2.2 ± 0.4	2.6 ± 0.8
S1	5.1 ± 0.7	2.4 ± 0.7
S2	6.7 ± 0.8	2.3 ± 0.7
S3	6.2 ± 0.2	3.2 ± 0.9

(6.2 ± 0.2) and S1 (5.1 ± 0.7). On an overall basis, the highest antibacterial activity was found in the bacterial isolates from the leaves of *C. procera* in comparison to the isolates from stems and roots (Tab. 1).

The selected strains were utilized against the pathogenic fungal strain, *A. flavus* (Tab. 1). Compared with the control the highest antifungal activity was shown by the isolated strain R1 (4.1 ± 0.6), followed by R2 (3.6 ± 0.4), R3 (3.0 ± 0.1), R4 (2.8 ± 0.5), and R5 (2.1 ± 0.1). The lowest antifungal activity was exhibited by R6 (1.8 ± 0.5). Similarly, leaves of *C. procera* also showed antifungal activities in

different concentrations; L3 was recorded as the dominant one (2.6 ± 0.8), followed by L2 (2.2 ± 0.7) and L1 (2.2 ± 0.4). The strains isolated from the stem of *C. procera* also showed varied antifungal activities (Tab. 1). The highest and most leading strain was S3 (3.2 ± 0.9), followed by S1 (2.4 ± 0.7), and S2 (2.3 ± 0.7) possessed the lowest antifungal activities and was considered as the best source of antifungal agents (Tab. 1).

Discussion

Endophytes have been studied for decades and reported to have a contribution to growth enhancement and plant health through several metabolic activities (Bilal *et al.*, 2018; Hamayun *et al.*, 2017).

Endophytes can play important beneficiary roles in host plant development under normal and stress conditions. Therefore, the role of endophytes in host plant life is very important that needs to be explored at physiological, biochemical, and molecular levels. In the present study, we have

CLB: negative control (media without extract); Ampicillin: positive control (media supplemented with commercial antibiotics); R1, R2, R3, R4, R5, R6: endophytes isolated from roots of *C. procera*; L1, L2, L3: endophytes isolated from leaves of *C. procera*; S1, S2, S3: endophytes isolated from stems of *C. procera*.

isolated 12 bacterial endophytes from the stem, leaves and roots of the *C. procera* plant. Out of the 11 isolated bacterial strains, 6 strains (R1, R2, R3, R4, R5 and R6) were isolated from the roots, 3 strains (L1, L2, L3) from the leaves, and 3 strains (S1, S2 and S3) from the stem. The extract of these bacterial endophytes was tested against the growth parameters of the rice plants.

The strain R1 was the potent strain that promoted root and shoot length and weight, which means that the strains can be used as a potent plant growth promoter. On the other hand, the strain L2 inhibited the rice plant growth, which means that the tested strain has the ability to release allelochemicals and can be used as a bio-control agent against weeds. Similar observations have been recorded in previous studies, where plant growth parameters were supported by the endophytes under normal as well as stressful conditions (Ismail *et al.*, 2020c; Muhammad *et al.*, 2019). This shows the importance of endophytes in sustainable agriculture in a continuously changing environment.

Plants contain minute amounts of the IAA, a phytohormone and free acid that helps in the plant growth promotion. Besides plant endogenous IAA, endophytes can also secrete IAA to support the host plants under normal as well as stress conditions. In the present study, the isolated strains from the stem, leaves and roots of the *C. procera* plant secreted different concentrations of IAA. The variability in the release of IAA in different concentrations by different strains might be due to the requirements of different precursors. For example, in the presence and absence of precursor tryptophan 66 bacterial strains were able to produce IAA in varying amounts (Phetcharat and Duangpaeng, 2012). Furthermore, the isolated strains from *C. procera* also produced flavonoids in appreciable quantities, especially the bacterial strain R6. However, the flavonoids contents were different in different bacterial

species, which reflects the variability and diversity of endophytes in the same plant species. Our results coincide with the results of (Etesami *et al.*, 2014). In fact, the presence of IAA and flavonoids in the extracts of isolated bacterial species confirms that they could be used as bio-fertilizer and bio-control agents. The endophytic strains thus have the ability to promote plant growth by secreting plant regulators and some other bioactive secondary metabolites, like flavonoids, to resist stresses (Ikram *et al.*, 2018).

There is an increase in the number of infectious diseases, including bacterial infections with various levels of drug resistance. This has resulted in the increasing use of natural products and a search for new antimicrobial drugs. Endophytes are one of the best candidates to explore for new drugs against degenerative diseases. Endophytes certainly contain numerous biologically active compounds, some of which have shown antimicrobial activities against pathogens (Ikram *et al.*, 2019). Similarly, in the current study, the isolated strains from different parts of *C. procera* exhibited antimicrobial activity. The extracts of some of the isolated strains showed the highest potency against the pathogenic microbes, while others showed very low activity. Such variability concerning the antimicrobial activity revealed the presence of a diverse group of endophytes in *C. procera* plants. Moreover, the antimicrobial activity of the extracts from the isolated strains also depends on the solvent system. The antimicrobial compounds in the crude extracts are; therefore, need to be separated by different solvent systems with varying polarity (Khan *et al.*, 2019). Antimicrobial activities of bacterial endophytes isolated from various plant species, such as *Aloe vera*, *P. tenuiflorus*, and *C. procera* has been reported to date (Akinsanya *et al.*, 2015; El-Deeb *et al.*, 2013; Mohamed *et al.*, 2019).

Conclusion

Endophytes are capable of promoting the plant's growth and protecting them from several biotic and abiotic environmental stresses. In the current study, we were able to isolate 12 strains of endophytic bacteria from *C. procera*, but 3 strains, i.e., R1, R4 and L2 can be used as plant growth promoters. Additionally, all the strains were able to show antibacterial activity against *E. coli*; whereas, the only strain R1 had the highest antifungal activity against *A. flavus* and can be used as a potent antimicrobial agent.

Ethics Approval: Our study did not involve any human, animal or endangered species.

Consent for Publication: No consent/approval at the national or international level or appropriate permissions and/or licenses for the study was required.

Availability of Data and Material: All the data are included in the manuscript.

Author Contribution: The authors confirm contribution to the paper as follows: Study conception and design: MH, SAK, IJL; data collection: MH, NK, MNK, MQ; analysis and interpretation of results: MH, AH, AI; draft manuscript

preparation: MH, AI, SAK; final editing: MH, AH, IJL. All authors reviewed the results and approved the final version of the manuscript.

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Conflicts of Interest: The authors declare that there is no competing interest of any nature related to this manuscript.

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