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EXPERIMENTAL INVESTIGATION OF PLANT GROWTH-PROMOTING QUALITIES BEARING ENDOGENOUS BACTERIA FROM ABIOTIC STRESS RESISTING HIGH ELEVATION PLANTS

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ABSTRACT

The microorganisms that live in the host are known as endophytes. They help in plant development by secreting numerous plant growth-promoting compounds like IAA, gibberellin, siderophore, and cytokinin. They also provide resistance to plants against many pathogen attacks. The present research focuses on isolating and screening PGP endophytic bacteria from six stress-bearing plants named *Commiphora wightii, Salvia rosmarinus, Lantana camara, Phoenix dactylifera, Haemerocallis fulva,* and *Abutilon indicum. Lantana camara, Phoenix dactyli- fera, Hemerocallis fulva, and Salvia rosmarinus* samples were collected from the Himachal Pradesh district Kangra village Noushera, *Commiphora wightii* from Chandigarh Botanical garden and *Abutilon indicum* from Jalandhar near city wastewater outlet. There were 50 isolates, i.e., eleven endophytic bacteria from *Commiphora wightii,* four from *Rosemary,* nine from *Lantana,*11 from *Phoenix dactylifera,* eight from *Haemerocallis fulva,* and seven from *Abutilon indicum* were isolated. Isolates obtained were further tested for IAA, gibberellin, cytokinin, and siderophore production. It was found that the three isolates, L1L2, G2R1, and DL3R2, isolated from *Lantana* leaves, *Commiphora wightii* roots, *and Hemerocallis fulva* roots, have shown IAA production. The maximum amount of IAA was produced by bacterial isolate DL3R2, i.e., 29.5 µg/ml at 10% tryptophan concentration. The maximum amount of gibberellic acid is 550 µg/ml produced by the isolate A1S1S. 16srRNA sequencing analysis has identified isolate A1S1S as *Pelomonas aquatica AIS1S* and DL3R2 as *Solibacillus silvestris DL3R2*. The isolates obtained can enhance plant growth and biofertilizers, thus suggesting an alternative approach to sustainable agriculture.

Key Words: Indole acetic acid (IAA) Endophyte bacteria Commiphora wightii Rosemary Haemerocallis fulva, Biofertilizer

1. Introduction

Endophytes are microorganisms that reside in the host tissue. They promote plant development by modifying soil chemistry or secreting substances stimulating plant development. The bacterial endophytes either directly encourage the growth of their host plants by manufacturing phytohormones such as cytokinins, gibberellins, and indole-3-acetic acid (IAA), abscisic acid (ABA), or indirectly stimulate the development of their host plants by producing exopolysaccharides (EPS), phosphate solubilization, forming siderophores and antimicro- bial. Furthermore, endophytes release a wide variety of secondary me- tabolites, including volatiles, that exert a significant

influence over the proliferation of phytopathogens and restrict their growth. Some of the most common bacterial genera found in plants are *Acinetobacteria*, *Pantoea*, *Bacillus*, and *Pseudomonas*, while Bacteroidetes, Firmicutes, Actinobacteria, and Proteobacteria are the most prevalent bacterial phyla [1]. Endophytes attach to the host cells through fimbriae, cilia, orflagella. Exopolysaccharides secreted by cells also help in attachment tothe root surface. Exopolysaccharides protect the cell and bind to heavy metals due to functional groups. EPS protects the bacterial cells againststress and helps to form biofilm [2]. Several studies mentioned that endophytes could be used as plant growth-promoting bacteria (PGPB) and an inoculant for integrated crop management [2–4]. *Bacillus* species isolated from orchids have been known to release IAA, which benefited plant growth [4]. Indole acetic acid plays a vital role in plant growth by encouraging apical dominance initiation of adventitious and lateralroots. Cytokinin helps in the division of cells. In contrast, gibberellin breaks dormancy and aids in plant elongation [5]. They also provide resistance to plants against many plant pathogenic viruses, bacteria, protozoans, fungi, and nematodes. They either destroy the pathogen cellwall by secreting various enzymes or release certain repelling chemicalsthat may prevent the pathogen from attacking the plants [6]. In addition, they also help the plants to tolerate different abiotic stress, such as varying levels of heavy metals, temperature variation, and salt concentrations [7]. Plant growth promotion and salt tolerance activity have been shown by the endophytic bacteria *Acinetobacter* and *Bacillus*species isolated from *Phyllanthus amarus* [8]. In order to address the abovementioned challenges and fulfill the desired objectives of agri- cultural production, there is an immediate requirement to find an effi- cient, environmentally friendly, and cost-effective alternative that promotes plant growth and yield. Native endophytic bacteria, which arethe most fundamental type of prokaryotic microbe, have the potential tobe new sources of the aforementioned PGP activities. The present research deals with the isolation and screening of IAA, gibberellin, and cytokinin-producing endophytes from six abiotic stress-bearing plants *Commiphora wightii*, *Salvia rosmarinus*, *Lantana camara*, *Phoenix dactyli- fera*, *Haemerocallis fulva*, and *Abutilon indicum.*

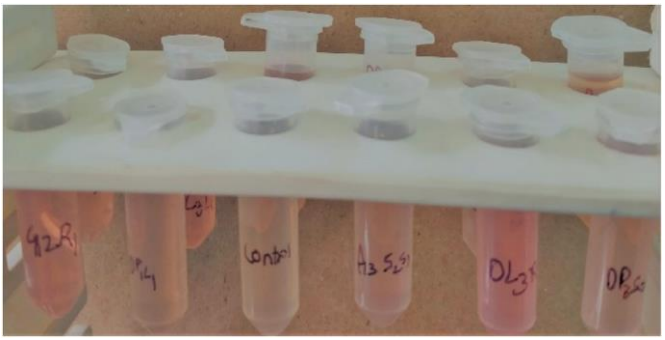


Fig. 1. Pink color production indicates the production of IAA.

addition, they also help the plants to tolerate different abiotic stress, such as varying levels of heavy metals, temperature variation, and salt concentrations [7]. Plant growth promotion and salt tolerance activity have been shown by the endophytic bacteria *Acinetobacter* and *Bacillus* species isolated from *Phyllanthus amarus* [8]. In order to address the abovementioned challenges and fulfill the desired objectives of agri- cultural production, there is an immediate requirement to find an effi- cient, environmentally friendly, and cost-effective alternative that promotes plant growth and yield. Native endophytic bacteria, which arethe most fundamental type of prokaryotic microbe, have the potential tobe new sources of the aforementioned PGP activities. The present research deals with the isolation and screening of IAA, gibberellin, and cytokinin-producing endophytes from six abiotic stress-bearing plants *Commiphora wightii*, *Salvia rosmarinus*, *Lantana camara*, *Phoenix dactyli- fera*, *Haemerocallis fulva*, and *Abutilon indicum.*

2. Methodology

2.1. Host plants

Lantana camara, Phoenix dactylifera, Hemerocallis fulva, and Salvia Rosmarinus samples were collected from the Kumau region district Nainital UK, India, Commiphora wightii from Chandigarh Botanical garden, Chandigarh, India and Abutilon indicum from Jalandhar, Punjab, India, near city waste water outlet (32.288067◦
N latitude 75.591223◦ E longitudes).

2.2. Screening and quantification of isolates

The plant's roots, stem, and leaves were cleaned with sterilized distilled water three rinses to remove dirt and adhere soil from the surface. Surface sterilization was accomplished by submerging the sample in ethanol (70%) for 1 min, followed by sodium hypochlorite (3%) for 3 min. Finally, the sample was rewashed in sterilized distilled water to eliminate any remaining chemicals from the sample surface [9]. Surface sterilized samples were pulverized in a sterile sucrose solution using a sterile mortar and pestle. They were then added to the nutrient broth and incubated at 30 2 ◦C for 24 h. The bacteria colonies were subcultured on nutrient agar plates until pure colonies were obtained. Fifty isolates were obtained after the purification process.

2.3. Indole acetic acid

All 50 isolates were subjected to an indole acetic acid production assay. Tryptophan broth (HM peptone 10 g/l, Sodium chloride 5 g/l, D-L tryptophan 5 g/l) was prepared, and the individual culture was inocu- lated in broth. All inoculated cultures in Tryptophan broth were incu- bated at 30 ± 2 ◦C for 24–48 h. Afterwards, the broth was centrifuged, and the supernatant was maintained to detect IAA produced in the su- pernatant. Salkowski's reagent was used to determine the IAA produced. Salkowski reagent was added to the supernatant and left at room tem- perature for 30 min. The appearance of pink color in the broth is char- acterized as IAA production in the supernatant [10,11]. Isolates positive for the indole test were subjected to different tryptophan concentrations (2–12%) for IAA quantification and optimization. Absorbance was recorded at 650 nm [12–14].

IAA standard curve preparation: from the stock pure IAA solution (Hi-Media, India), dilutions of standard indole acetic acid were prepared at concentrations of 10, 20, 30, 40, and 50 µg ml—1 to determine the quantity of indole acetic acid produced by the endophytic bacteria.

2.4. Gibberellic acid

Bacterial cultures were grown in Nutrient Medium for five days at 32 2 ˚C before being centrifuged at 10,000 rpm for 15 min. The pH was kept constant during the process at 2.5. Ethyl acetate was used to extract filtrate for gibberellic acid detection. Dilutions of standard gibberellic acid were prepared with a concentration range from 100 to 600 µg/ml. Absorbance was recorded at 254 nm. TLC was carried out using three solvent combinations, i.e., isopropanol: ammonia: water in a ratio of 16:3:1. The greyish fluorescent dots were developed after spraying ethanolic sulphuric acid (95:5) and then heating the sample at 120 ◦C for min [15–17]. The following formula was used to calculate the rf value:

Rf = distance travelled by compound /Distance travelled by the solvent

2.5. Siderophore

Chrome-azurol S (CAS) medium was used to confirm the siderophore production potential of bacterial isolates. Bacterial culture after 24 h incubation was inoculated on CAS agar and incubated for 48–72 h at 30 2 ◦C. Change of CAS agar medium from blue to orange or yellow around bacterial growth confirmed siderophore production [18,19].

2.6. Cytokinin

0.01% thiamine, 0.2% casamino acids, and 2 pg of biotin were added to 1000 ml sterilized distilled water M9 media (Hi-media, India). The bacterial culture was inoculated and incubated at 30 2 ◦C, 160 rpm for five days. Standard was prepared with kinetin varied concentration range from 0 to 50 µg ml—1. Quantification was done by taking absor- bance spectrophotometric ally at 665 nm at 72, 96, and 120 h [15].

2.7. 16s RNA analysis

The EXpure Microbial DNA isolation kit (Bogar Bio Bee stores Pvt Ltd.) was employed to extract DNA from bacterial samples and 16S rRNA gene (rDNA) bacterial primers 27F 5' (AGAGTTTGATCTGGCT- CAG 3') and 1492R (5′ TACGGTACCTTGTTACGACTT 3′) was used. Isolated DNA (5 µL) was added in 25 µl of PCR reaction solution (1.5 µL of Forward Primer and Reverse Primer, 5 µL of deionized water, and 12 µl of Taq Master Mix). PCR was performed. Single-pass sequencing was conducted on each template using the 16s rRNA universal primers mentioned above. The fluorescent-labelled fragments were purified from an ethanol precipitation protocol by unincorporated terminators. The samples were resuspended in distilled water and subjected to elec- trophoresis in an ABI 3730xl sequencer. The 16s rRNA sequence was blasted using the NCBI nBlast similarity search tool. The phylogeny analysis of the query sequence with the closely related sequence of blast results was performed, followed by multiple sequence alignment using MEGA X software [20,21].

2.8. Data analysis and interpretation

All the data were analyzed using SPSS software. One-way ANOVA and Post HOC test were applied. All the observations about the con- centration of phytohormones produced were taken as per the p value < 0.05.

3. Results

In the study, a total of 50 endophytic bacteria were isolated. Of these, 11 were isolated from the *Commiphora wightii* plant, four from the *Salvia rosmarinus* plant, nine from the *Lantana camara* plant, eleven from the *Phoenix dactylifera* plant, eight from the *Haemerocallis fulva* plant, and seven from the *Abutilon indicum* plant. All plants were collected from Himachal Pradesh, Chandigarh, and Jalandhar, India. The nomenclature of isolates was carried out as mentioned below, The first letter denotes the Plant name, the second letter denotes

the part of the plant from which the isolate has isolated such as 'L' denotes the leaves, 'S' stands for stem and 'R' stands for root, and 1,2,3 stands for isolate number from an individual plant for example in R1L1, the R1 stands for the first sample of Rosemary, and 'L' stands for Leaves, and '1′ stands for isolate number 1. Bacterial isolates were examined for biochemical tests. Furthermore, they were subjected to plant growth promotion attributes to determine Indole acetic acid (IAA), gibberellic acid, cytokinin, and siderophore concentration in the culture broth. The IAA was quantified using a UV–Vis spectrophotometer at 650 nm, gibberellic acid was measured at 254 nm, and cytokinin was measured at 665 nm. Among 50 isolates, only six isolates L1L2 isolated from *Lantana* leaves, G2R1from *Commiphora wightii* roots, DL3R2, DL2R1, DL3L1 from *Hemerocallis fulva* roots and leaves, and DP2L1from *Phoenix dactylifera* leaves have shown positive indole test (Fig. 1). The amount of IAA produced by the isolates is shown in Table 1(a), provided below.(See Table 1b). Since the p-value is less than 0.05, we reject the null hypothesis, meaning a statistical significance exists at different concentrations. Fig. 2 shows that the maximum amount of IAA produced was 29.5 µg/ml by the *Solibacillus silvestris DL3R2* isolated from *Hemerocallis fulva* (Day lily) at 10% tryptophan concentration. There is a decrease in IAA concentration by all the isolates at 12% tryptophan concentration except G2R1, which produces 28 µg/ml of IAA. The highest amount of gibberellic acid produced was 550 µg/ml by the isolates AIS1S and DL3R2 isolated from *Abutilon indicum* stem and *Hemerocallis fulva* root, respectively (Table 2) Identification of Gibberellin by Thin layer Chromatography.

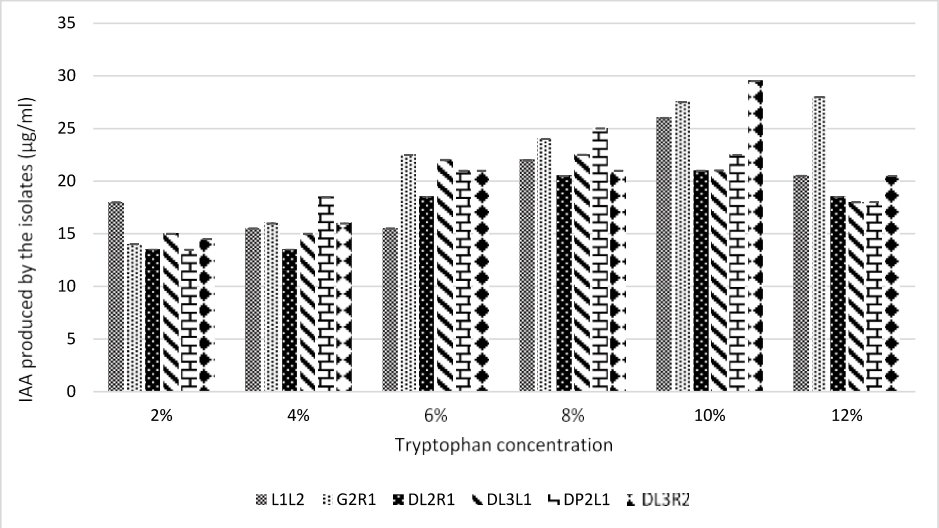


Fig. 2. Amount of IAA produced by the isolates in µg/ml at different tryptophan concentrations with standard deviations.

Table 2

Quantification of Gibberellic acid produced by the isolates, n = 3.

Bacterial isolate	Amount produced (µg/ml)[a]
G2R2	420 ± 1.587
R3L1	420 ± 1.582
L1L2	430 ± 1.71
DP1S1S2	520 ± 2.218
R1L1	540 ± 2.259
A1S1S	550 ± 2.512
DL3R2	550 ± 1.842

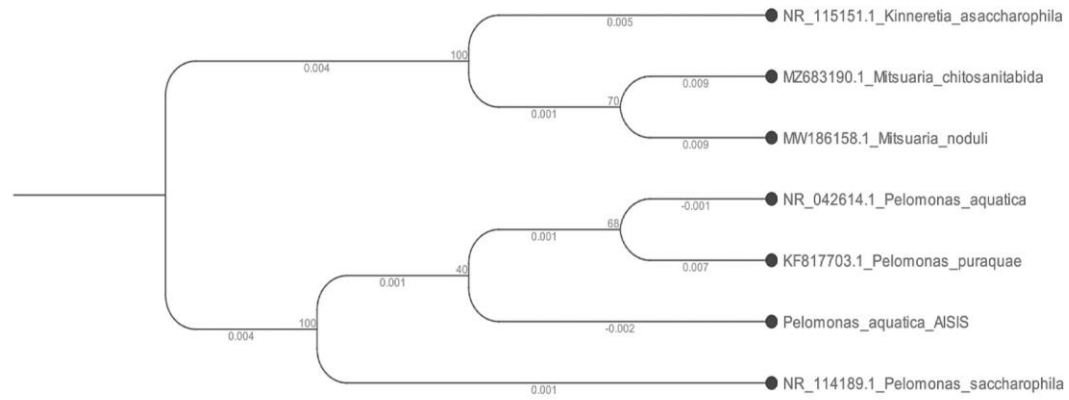
[a] Values in columns are mean ± Standard deviation.

3.1. 16s rRNA analysis

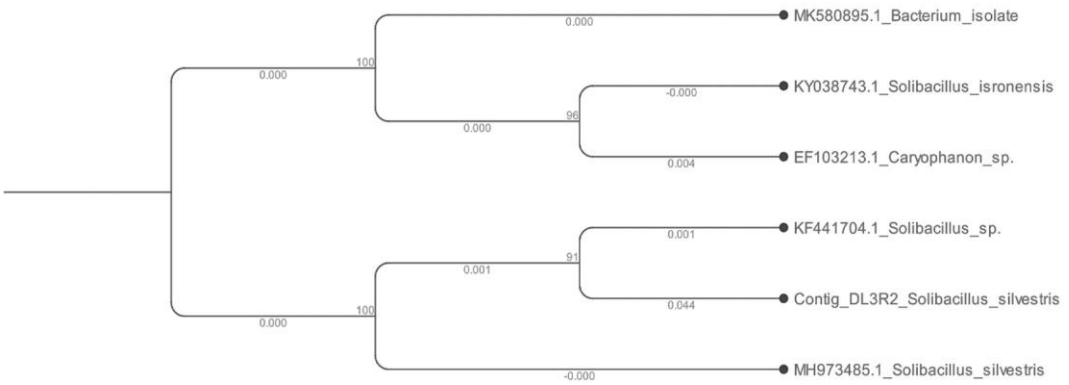
The analysis has shown that isolate A1S1S shows the highest homologies of 100.0% and 99.2% with those of *Pelomonas aquatica* CCUG 5257516S (NR 042614.1), and *Pelomonas aquatica* CCUG 52507 (AM501434.1). The strain was identified as *Pelomonas aquatica* A1S1S and phylogenetic tress is shown in Fig. 4(a). The isolate DL3R2 has shown the highest homologies of 93.61% and 93.53 with those of *Solibacillus strain* LQ5 and *Solibacillus silvestris strain* IADCACS25.

4. Discussion The endophytic microbial community is active and able to spread effectively across all plant tissues. This type of colonization is advantageous for a plant since the colonized community can communicate effectively and interact despite the plant's unfavourable environment [5]. This results in an increase in plant growth and maximum protection from phytopathogens, an improvement of stress resistance, and an acceleration of the production or synthesis of bioactive substances that are of importance. Various findings have indicated that plant tissue-associated bacterial strains can stimulate plant growth and development via photostimulation, biofertilization, and biocontrol [8, 12]. Several genera of bacteria, including *Pseudomonas*, *Pantoea*, *Bacillus*, *Serratia*, *Enterobacter*, *Azospirillum*, and *Paraburkholderia*, are advantageous to the development of plants [17]. Endophytic microbes promote plant growth by regulating plant physiological processes such as N2 fixation, phosphate solubilization, and the synthesis of ammonia, siderophores, phytohormones, and hydrolytic enzymes [21]. Plant growth-promoting traits and endophytic bacteria are directly or indirectly linked. These bacteria colonies in the host tissues promote plant growth and protect them against many pathogen attacks. They penetrate the endosphere and adapt to the environment [22]. Root endophyte *Piriformospora indica* synthesized auxin by IAA biosynthetic pathway [23]. The IAAs produced by endophytes are thought to have a significant role in controlling plant development. According to Khan et al. [24], the endophyte *Sphingomonas* isolated from the foliar region of *Tephrosia apollinea* enhanced the growth rate of tomato plants by producing indole acetic acid at a concentration of 11.23 µg/ml. It was also determined by GC-MS analysis that the bacteria *Staphylococcus epidermidis* RWL-7, *Micrococcus luteus* RWL-3, *Pantoea dispersa* RWL-3, and *Micrococcus yunnanensis* RWL-2 all produced IAA rage from 11.50 to 38.80 g/ml. Endophytes considerably improved the key growth-promoting attributes of rice plants after being inoculated, including protein content, chlorophyll, shoot and root length, and dry biomass [25]. The endophytic fungus *Falciphora oryzae* supported the proliferation of lateral roots and lowered the height of the primary roots [26].

Fig. 4. Phylogenetic tree of isolated bacterila endophytes **(a)** *Pelomonas aquatica* A1S1S, **(b)** *Solibacillus silvestris* DL3R2.



(a)



(b)

normal and salt-affected conditions, the fungal endophytes *Porostereum spadiceum* boosts the germination of seeds and total chlorophyll to recover soybean development [27]. Numerous types of endophytic fungi, such as *P. spadiceum*, *Pochonia chlamydosporia*, *Rhizopus stolonifer*, *Penicillium corylophilum*, *P. glomerata*, *Paecilomyces formosus*, and *Aspergillus flavus* is the first endophyte to produce gibberellic acid and be engaged in phytostimulation. However, the vast majority of endophytic fungi that make similar compounds are Ascomycetes [24]. Cytokinins are a class of plant hormones that play a role in various vital operations, including apical dominance, plastid maturity, cellular growth and diversification, seedling growth, preventing senescence, and signalling between plants and pathogens [28]. Bacterial endophytes *Arthrobacter*, *Stenotrophomonas*, *Sphingomonas*, Bacillus, and *Pseudomonas* were identified from humic-treated cucumber plants, and they produced various cytokinins with concentrations of more than 30 pmol/ml. These cytokinins included *cis*-zeatin cytokinin, riboside type zeatin, isopentyladenine, and iso-pentenyladeno [29]. Our study focuses on isolating PGP endophytic bacteria from abiotic stress-bearing plants and examines the potential for IAA, gibberellic acid, cytokinin, and siderophore production by these endophytes. gibberellic acid; however, they were negative for siderophore production. The highest amount of IAA is produced by the *Solibacillus silvestris* DL3R2 isolated from *Hemerocallis fulva* roots at 10% tryptophan concentration. The amount of IAA produced was 29 µg/ml. The isolates have shown a sharp decrease in IAA at 12% tryptophan concentration. It has been reported that the endophytes can potentially increase the IAA concentration with an increase in tryptophan concentration ranging from 10 to 60 µg/ml [30]. In the case of gibberellic acid, *Pelomonas aquatica* A1S1S isolated from *Abutilon indicum* were found to be the maximum gibberellic acid-producing

isolates. On TLC analysis *Pelomonas aquatica* A1S1S has also shown the highest rf value, i.e., 0.74 ± 0.014. Our results are supported by the studies showing that gibberellin extracted from *Pseudomonas* sp. With TLC analysis has shown rf values of 0.78 and 0.79 [24]. DL3R1, L1L1, and G1R1 isolated from *Hemerocallis fulva* roots, *Lantana* leaves, and *Commiphora wightti* roots have shown maximum cytokinin production after 120 h of incubation and the amount produced was 35 µg/ml, 35 µg/ml, and 33.5 µg/ml, respectively. DL3R2 has shown a slight decrease in cytokinin production after 120hrs of incubation. The L3L2 isolate has shown the highest rf value, 0.890 ± 0.068, whereas L1S2 shows the lowest. Several studies are associated with endophytes and their PGP activities, showing that they can help plants deal with the change in environmental conditions and help plant growth promotion. Endophytic bacteria produce many compounds that promote plant growth and tolerate stress, such as salt and heavy metal. Endophytic bacteria producing IAA is known to increase the Maize's seed germination and root elongation [31]. Similarly, IAA and gibberellin-producing endophytes have alleviated the effect of NaCl and promoted rice plant growth [32]. Some strains of endophytic bacteria isolated from Phoenix dactylifera have been associated with improved IAA and ACC activity with increased salt concentration. This indicates that salinity can influence the IAA and ACC deaminase activity and may improve the nutrient uptake and development of plant growth in situations with high levels of salt stress [33]. *Achromobacter xylosoxidans T16, Achromobacter insolitus R15b, and Pantoea agglomerans T12* isolated from sugarcane possess nitrogen fixation, phosphate solubilization, and indole biosynthesis activity. They can be used as biofertilizers [34]. Endophytic bacteria strain *Pseudomonas, Stenotrophomas, and Achromobacterer* isolated from *A. hybridus, S. lycopersicum,* and *C. maxima* plants produce IAA, HCN, ammonia and possess the antifungal property that may promote the plant growth as well protects it against diseases [35,36].

4. Conclusion

All bacteria isolated from six plants were screened for IAA, gibberellin, cytokinin, and siderophore. The results have shown that a few strains could produce IAA and gibberellin, but none have shown a positive response to the siderophore test. *Solibacillus silvestris DL3R2* has shown maximum IAA production at 10% tryptophan concentration. *Pelomonas aquatica A1S1S* produces the highest gibberellic acid, i.e., 550 µg/ml. DL3R1 has produced the highest cytokinin amount, i.e., 35 µg/ml after 120hrs incubation. This study provides information about exploiting these bacterial isolates for plant growth promotion or as a bio inoculum for biofertilizer production, thereby providing an alternative way for sustainable agriculture.

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