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Research Article

Integron Mediated Multiple Heavy Metal and Antibiotic Resistance in Plant Growth Promoting Epiphytic Bacteria

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Abstract: The present study was conducted to investigate the co-selection of antibiotic and heavy metal resistance in epiphytic bacteria isolated from *Withania somnifera, Ficus benghalensis, Olea europaea* and *Aloe vera*. Thirty epiphytic bacterial strains were isolated. Six isolated strains were selected and observed to have significant multiple heavy metals and antibiotics resistance. Single and synergistic effect of heavy metals and antibiotics constantly boosted the growth rate of selected bacterial isolates. Inoculation of these epiphytic bacteria caused increment in seedling, shoot and root length upto 58-70%, 25-37%, 87-125% respectively, while there was an increase in number of leaves upto 25-50% of *Triticum aestivum.* These epiphytic bacteria exhibited high extracellular antioxidant potential with rise in DPPH (2, 2-diphenyl 1-picrylhydrazyl) scavenging ability (33-59%) and phenols concentration (78-173 µg/ml). Phylogenetic analysis revealed 99-100% similarity of these bacterial strain AN1 *(Staphylococcus pasteuri)*, AN2 *(Microbacterium paraoxydans)*, BG4 *(Pseudomonas azotoformans)*, BG6 *(Staphylococcus haemolyticus)*, OL21 *(Staphylococcus haemolyticus)*, and AV2 (*Paenobacillus lactis)*. Sixty-six percent of these bacteria carried *IntI1* gene having similarity with *XerC* integrase/recombinases superfamily conserved domains. Our findings suggested that existence of *IntI1* gene in epiphytic bacterial genome helps in their survival under stress environment.

Keywords: Epiphytic Bacteria, Plant Growth Promotion, Antioxidant Activity, DPPH Activity, *IntI1* Gene.

1. INTRODUCTION

The phyllosphere, a distinctive ecological niche, undergoes significant perturbations in response to variations in environmental parameters, including temperature, UV exposure, and relative humidity. Nutrient availability plays a pivotal role in governing the colonization potential of symbiotic epiphytic microbial communities within the phyllosphere, which, in turn, influences plant fitness and growth. Effective colonization of these symbiotic epiphytic microbial communities is intricately linked to nutrient availability. Epiphytic microorganisms exhibit a remarkable ability to thrive in challenging environmental conditions, characterized by limited nutrients, temperature fluctuations, UV radiation exposure, elevated atmospheric heavy metal concentrations, and exposure to insecticidal agents. Microorganisms engage in passive diffusion mechanisms to leach inorganic ions and organic acids surfaces [1]. Epiphytic bacterial populations significantly contribute to plant by rapid multiplication on plant surfaces, supplying nutrient-rich environment which is rich in carbon and nitrogen sources, and giving protection against pathogenic bacteria and stressful conditions. The presence of these bacteria is primarily determined by nutrient exchanges between host plants and the microbial communities [2]. Epiphytic bacteria provide a range of advantageous contributions to host plants, comprising the augmentation of nutrient accessibility, the synthesis and orchestration of plant growth-regulating hormones, the promotion of heightened plant growth in the presence of stressors, the biosynthesis of antibiotics and lytic enzymes aimed at establishing a nutrient-deprived environment for plant pathogens, and fortification against the incursion of pathogenic organisms [3]. However, the functional, structural, and adaptive aspects of epiphytic microbial communities residing

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on host plant surfaces remain largely unexplored. The secretion of secondary metabolites represents an additional determinant affecting the dispersion of bacteria on plant surfaces, potentially conferring antibacterial characteristics against diverse plant pathogens [4].

The microbiome within the phyllosphere on leaves is directly influenced by the application of antibiotics, as the unintended dissemination and runoff of these substances result in the deposition of antibiotics within the soil. This, in turn, affects the integron-related *IntI1* gene in epiphytic bacteria and the rhizosphere microbiomes [5]. Natural antibiotic resistance genes are anticipated to evolve alongside bacterial antibiotic production functions as a means of self-protection. These mechanisms include hindering antibiotic mobility to target sites, modifying antimicrobial compounds, preventing the active transport of antimicrobial compounds, synthesizing new proteins insensitive to antibiotics, mutating the target site, and protecting the target site [6].

Notably, antibiotic-resistant bacteria are often resistant to heavy metals as well. This dual resistance entails processes, including shifts in membrane permeability, adjustments in the presence of heavy metals and antibiotics, alterations in target sites, and the acquisition of heavy metals and antibiotics [7]. The strong association between antibiotic and heavy metal resistance arises from co-resistance and cross-resistance processes. Recent studies have highlighted the presence of antibiotic resistance not only in bacterial genomes but also in plasmids in heavy metal-contaminated environments [8]. Integrons, featuring integrase (*IntI1*) gene-encoded site-specific recombinases, play a critical role in transferring antibiotic resistance genes in animal and human pathogens and accumulating multiple antibiotic resistance genes into a multi-resistance element [9].

Bacteria represent promising candidates for bioremediation, given their proficiency in detoxifying heavy metals. Microbial remediation, employing microbes for the accumulation and detoxification of heavy metals, stands as a leading method for this purpose [10]. Heavy metal like Chromium (VI) and zinc (II), contribute to elevate reactive oxygen species and oxidative cell impairment during detoxification. Consequently, the combined action of these metals significantly impacts cellular antioxidant properties and the persistence of heavy metal and antibiotic-resistant epiphytic bacteria in host plants [10]. Under such challenging conditions, these bacteria engage in long-term interactions with host plants, promoting plant growth [7]. Recent research has unveiled the potential of plant growth-promoting epiphytic bacteria to enhance the growth and yield of various cereal crops, including wheat [11, 12]. Without the presence of these beneficial epiphytic bacteria, plants would be less competitive against plant pathogens and exhibit reduced tolerance to stressful environments [13].

Recent studies showed that the role of exopolysaccharides (EPS) production as an adaptation mechanism of epiphytic bacteria to safeguard plants against dehydration due to the high-water maintenance capacity of EPS [14]. Additionally, bacterial production of hydrogen cyanide (HCN) provide protection to plants from bacterial pathogens. Epiphytic bacteria also enhancing the host plant growth by solubilization of phosphate, rendering it in a soluble form that is easily taken up by the host plant. Present study is focused on the investigation of the relationship between multiple heavy metals and antibiotic resistance patterns, as indicated by the presence of the integron-related *IntI1* gene, in epiphytic bacterial isolates from selected medicinal plants.

2. MATERIALS AND METHODS

2.1. Sample Collection of Medicinal Plants and Isolation of Epiphytic Bacteria

From the Botanical Garden of University of the Punjab, Lahore, Pakistan Medicinal plants (*Withania somnifera, Ficus benghalensis*, *Oleo europeae* and *Aleo vera*) were selected. Plants were collected with leaves and stems. Sterile polythene bags were used to carry the plant materials to the laboratory and plants were processed within 24 h to avoid the chances of contamination. Epiphytic bacteria were isolated by using stem-leaf-imprinting agar and serial dilution method. Thirty morphologically distinct bacterial colonies were selected from plates of both isolation methods after 48 hours of incubation at 37 °C. Selected isolates were further purified and stored at 4 °C. Selected bacterial isolates were observed for their morphological (Gram staining, colony and

cell morphology, motility test) and biochemical (oxidase, catalase, DNase, starch hydrolysis, TSI, urease, indole, Methyl Red (MR)-Voges Proskauer (VP) and gelatin liquefaction tests) characteristics. Bacterial isolates were identified by comparing 16S rRNA gene sequencing using BLAST against NCBI database. The nucleotide sequences were deposited in the NCBI GenBank to obtain accession numbers. The evolutionary relationships were assessed using MEGA 7 software and neighbor-joining method was used for the construction of phylogenetic tree. Effects of three different pHs (5, 7 and 9), temperatures (28 °C, 37 °C and 46 °C), carbon sources (glucose, fructose, sucrose, and lactose) and nitrogen sources (Peptone, Yeast extract, NH_4Cl, and KNO_3) on growth of bacterial strains were studied for 96 hours [15].

2.2. Heavy Metal and Antibiotic Resistance Profiling of Epiphytic Bacteria

Selected epiphytic bacterial isolates were screened against five heavy metals (K_2CrO_4, $ZnCl_2$, $PbCl_2$, $CuSO_4$ and $NiCl_2$) and two antibiotics of different classes (ampicillin and streptomycin) by agar plate dilution method [7]. Bacterial strains were grown on Luria Broth (LB) agar supplemented with variable concentrations (K_2CrO_4: 100 to 120000 µg/ml, $ZnCl_2$: 100 to 1400 µg/ml, $CuSO_4$ and $NiCl_2$: 100 to 1600 µg/ml, $PbCl_2$: 100 to 5600 µg/ml ampicillin: 100 to 2600 µg/ml, streptomycin: 100 to 1500 µg/ml) of selected metal and antibiotics and incubated at 37 °C for 48 hours. Maximum Tolerable Concentration (MTC) was determined. Six epiphytic bacteria were selected for further study on the basis of their high heavy metal and antibiotic resistance pattern.

2.3. Response of Epiphytic Bacteria in Single and Synergistic Conditions

Growth response under single and synergistic conditions for antibiotics (ampicillin and streptomycin) and heavy metals (Cr and Zn) were observed for selected epiphytic bacteria. LB broth was supplemented with 500 µg/ml of antibiotic and heavy metal in single and combination were inoculated with the respective bacteria. Incubations were carried out at 37 °C. Samples were withdrawn under sterile conditions after every 24 h for up to 15 days and optical density (OD) was measured at 600 nm.

2.4. Analysis of Plant Growth Promoting Characters in Epiphytic Bacteria

Solubilization of phosphate salt, HCN and auxin production tests were performed to determine the plant growth promoting potential of bacterial strains. For determination of phosphate solubilization potential of bacteria, Pikovskaya's (PVK) agar medium was used. Lorck method was followed for observing HCN production ability by bacteria and IAA (Indole-3-acetic acid) production by bacterial strains was estimated by using Salkowski's method [16].

2.5. Plant Microbe Interaction (PMI) Studies

In this experiment, healthy cash crop *Triticum aestivum* (FSD-08) was selected due to its economic importance and seeds were bought from Punjab Seed Centre, Lahore, Pakistan. Plate method was used to perform this experiment under controlled conditions of lab by following Mustafa *et al*. [17] with some modification. Different growth parameters of *Triticum aestivum* seedlings were recorded after two weeks of seed germination.

2.6. EPS Production by Isolated Epiphytic Bacteria

Selected bacterial strains were analysed for EPS production in the presence of LB media at optimal temperature (37 °C) for one week. Ice cold ethanol precipitation method was used for the extraction of EPS. The total EPS content was calculated by subtracting the dry weight of EPS from its fresh weight [18].

2.7. Preparation of Extracellular and Intracellular Epiphytic Bacterial Extracts for Antibacterial and Antioxidant Profiling

Solvent ethyl acetate was used to prepare extracts of extracellular and intracellular epiphytic bacteria. Epiphytic bacteria which were previously selected cultured in LB and incubated for 24 hours at 37 °C Isolation extracellular metabolites, cell-free supernatants were combined with ethyl acetate in a 1:1 ratio. The organic layer was evaporated using a rotary evaporator at 37 °C and re-suspended in 20% Dimethyl sulfoxide (DMSO) for future use. In order to extract intracellular bacterial metabolites, ethyl acetate was also used. The bacterial pellet

was sonicated to break the cells open. The resulting lysed cell suspension was centrifuged at 8000 g for 10 minutes, and the supernatant was dried in the rotary evaporator at 37 °C. The dried substance was weighed and re-suspended in 20% DMSO for later use. All extracts of the selected bacteria were tested for antibacterial activity using the agar well diffusion method. *Bacillus* KC881030 and *Pseudomonas* KC881031 test cultures were spread onto Mueller-Hinton (MH) agar plates. Ampicillin at a concentration of 30 µg/ml was the standard, while DMSO served as the control. The plates were then incubated at 37 °C for 24 hours and the diameter of inhibition zones (mm) was measured. The assessment of antioxidant capacity of extracellular and intracellular ethyl acetate extracts of epiphytic bacteria encompassed three techniques: catalase examination, DPPH (2, 2-diphenyl 1-picrylhydrazyl) test, and TPC (total phenolic content) determination. Catalase is an enzyme crucial for combating oxidative stress in bacteria. The catalase activity in specific bacteria was assessed by reacting hydrogen peroxide with a supernatant sample and recording the absorbance at 240 nm for 60 seconds at 15-second intervals. Intra and extracellular extracts of epiphytic bacteria in ethyl acetate solvent were analyzed by the DPPH radical scavenging activity. In presence of stress conditions phenolics compounds are produced by bacteria. The Folin-Ciocalteu method was employed to determine the total phenolic content in extracellular and intracellular extracts of isolated epiphytic bacteria [19].

2.8. Amplification of *IntI1* Gene in Epiphytic Bacteria

Genomic DNA isolation was performed by using Thermo Scientific Gene JET Genomic DNA Purification Kit according to manufacturer instructions. *IntI1* gene amplification was carried out on gradient PCR thermocycler machine amplification using following primers: *IntI1* F (5' CCTCCCGCACGATGATC 3') and *IntI1* R (3' TCCACGCATCGTCAGGC 5'). The program of 30 cycles was run by setting annealing temperature 58 °C. Amplified PCR products were examined by gel electrophoresis. *IntI1* gene was sequenced and different NCBI bioinformatic tools were used for analysis and accession number were obtained by submitting sequences to NCBI GenBank. BioEdit software was used for multiple alignment of gene sequences and phylogenetic tree was constructed by MEGA 7 software using neighbor-joining method [20].

3. RESULTS

3.1. Isolation of Epiphytic Bacteria

Total 30 morphologically different epiphytic bacterial strains were isolated from collected plants by serial dilution method. All selected epiphytic bacteria exhibited highly diverse morphological and biochemical characteristics. All bacterial colonies were round in shape with entire margins except AV2 (irregular) and smooth in texture except AN1 and AV2 (mucoid). Color of bacterial colonies was off-white (AN1, BG4, AV2), white (BG6, OL21), yellow (AN2) with opaque (AN2, BG6, OL21), translucent (BG4, AV2) and transparent (AN1). Most of the strains were gram positive except BG4, AV2 (gram negative); 50% bacteria (AN1, BG4, OL21) were cocci and 50% (AN2, BG4, AV2) were rods. Except AN2 (spore former) and non-motile except AV2 (motile), all bacteria were non-spore formers. Biochemical characterization revealed that 83% bacteria showed positive results for catalase test; 66% positive for starch hydrolysis and VP test; 50% for DNase, methyl red and gelatin liquefaction test; 33% for oxidase and 16% for urease, MR-VP and gelatin liquefaction test. The genetic analysis revealed that the 16S rRNA gene sequences of bacterial strains AN1, AN2, BG4, BG6, OL21, and AV2 exhibited high similarity (ranging from 99 to 100%) with reference strains and accession numbers were obtained (Table 1).

For growth optimization of isolated bacteria, strain AN2 and OL21 preferred to grow at pH 5 whereas, strain BG4 and AV2 grew best at pH 7. Bacterial strain AN1 and BG6 showed best growth at pH 9. Three bacterial strains (AN2, BG6 and OL21) exhibited optimum growth at 46°C while strain AN1 and AV2 preferred 37°C. Only strain BG4 showed good growth at 28°C. Glucose was the best carbon source for the growth of strain BG4 and OL21 but sucrose was best for strain BG6 and AV2. Optimized growth patterns of epiphytic bacteria AN1 and AN2 was observed when media supplemented with lactose and fructose. Peptone was found as best nitrogen source for the growth of all bacterial strains.

Table 1. Bacterial identity along with accession numbers and potential plant growth promoting activities of selected epiphytic bacteria.

Bacterial isolates	Nearest relative	Accession no.	Query cover	Identity	Plant growth promoting activities
AN1	*Staphylococcus pasteuri*	MK875469	98%	99.83%	IAA
AN2	*Microbacterium paraoxydans*	MK875470	99%	99.73%	HCN, IAA
BG4	*Pseudomonas azotoformans*	MK875666	99%	99.80%	HCN, IAA
BG6	*Staphylococcus haemolyticus*	MK874945	96%	100%	PS, HCN, IAA
OL21	*Staphylococcus haemolyticus*	MK875667	99%	99.87%	PS, HCN, IAA
AV2	*Paenobacillus lacti*s	MK874992	99%	99.93%	HCN, IAA

IAA: Indole acetic acid, HCN: Hydrogen cyanide, PS: Phosphate solubilization

3.2. Resistance Profiling of Selected Epiphytic Bacteria

About 20% of bacterial isolates were found to be highly resistant to heavy metals and antibiotics with MTC ranging from 1000-100000 µg /ml for heavy metals and 500-2500 µg/ml for antibiotics. Four bacterial strains (AN1, AN2, BG4 and BG6) exhibited highly diverse resistance pattern to almost all tested heavy metals and antibiotics. Strain AV2 showed resistance to just two heavy metals and two antibiotics tested. Bacterial isolates BG6 and OL21 revealed 80000-100000 µg/ml MTC of chromium whereas AV2 and AN2 were able to tolerate 2500 µg/ml and 1400 µg/ml of ampicillin and streptomycin, respectively (Table 1, supplementary data).

3.3. Synergistic Response of Selected Epiphytic Bacteria towards Multiple Metal and Antibiotics Stress

The two specific heavy metals (Cr and Zn) and antibiotics (ampicillin and streptomycin) were selected due to high resistivity of selected strains. For this, bacterial isolates were exposed to ampicillin, chromium, and zinc, both individually and in combination, at a concentration of 500 µg/ml. The presence of single and combined heavy metal and antibiotic stress distinctly influenced the growth of the selected epiphytic bacterial isolates. The findings featured that these epiphytic bacterial strains possess strong adaptability to both antibiotic and heavy metal stress, whether encountered singly or in combination, illustrating their impressive resistance patterns (Figure S1 supplementary data).

3.4. Screening of Epiphytic Bacteria for Plant Growth Promoting Characteristics

In this study, 33% of selected bacterial isolates (BG6 and OL21) had phosphate solubilization ability and 83% showed HCN production except AN1. All bacterial isolates had auxin production ability but highest auxin production was observed by two strains (AN2 and AV2) that was 17 and 28 mg/ml, respectively (Table 1).

3.5. Plant Microbe Interaction (PMI) Studies

Inoculation of four epiphytic bacterial isolates (AN1, AN2, BG4, and AV2) enhanced the percentage seed germination of *Triticum aestivum* up to 100%. Inoculation of these bacterial isolates increased seedling length, shoot length and root length up to 58-70%, 25-37%, 87-125%, respectively; while there was an increase in number of leaves up to 25-50% and roots of *T. aestivum* seedlings as compared to control (Table 2, supplementary data). It was observed from this experiment that epiphytic bacterial isolates had a plant growth promoting potential along with the antibiotic and heavy metal resistance.

3.6. Exopolysaccharides Production by Selected Epiphytic Bacteria

Among six epiphytic bacterial isolates, two strains (BG4 and AV2) produced maximum quantity of EPS ranging from 0.5-0.7 g/L after 7 days of incubation. Texture of EPS of both strains was spongy in wet form and hard in dry form whereas color of EPS was gray and brown, respectively (Figure. 1).

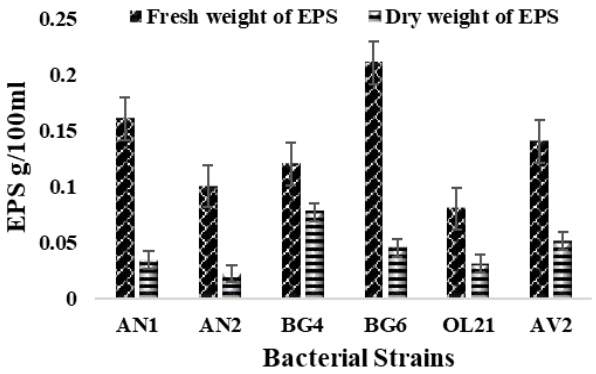

Fig. 1. Quantification of exopolysaccharides (EPS) produced by selected epiphytic bacteria.

3.7. Antibacterial and Antioxidant Activity of Epiphytic Bacteria

All selected bacterial isolates showed antibacterial activity by extracellular extract with 4-6mm inhibition zone against gram positive test bacteria while AN2 strain exhibited 6mm inhibition zone. But, antibacterial activity by intracellular extract was exhibited by only two strains (BG4 and AV2) with 4mm inhibition zone against gram positive test strain. Whereas, none of the selected bacteria showed antibacterial activity against gram negative test strain. Catalase activity profiling, DPPH (2, 2-diphenyl 1-1picrylhydrazyl) and total phenolic content (TPC) were the three methods used for the determination of intracellular and extracellular antioxidative property of epiphytic bacterial isolates. Catalase activity estimation of cell-free bacterial suspension revealed 50% of bacterial isolates showed maximum extracellular catalase activity with OD (0.120 nm) in AN2, (0.106) in OL21 and (0.178) in BG4 (Figure 2(a)). All selected bacterial isolates exhibited significant extracellular DPPH scavenging potential with 33-59% increase in DPPH reduction ability as compared to control. While, only one strain BG6 gave 29% intracellular DPPH scavenging activity (Figure 2(b)). Estimation of phenol concentration revealed that highly significant TPC of 78-173 µg/ml was found in extracellular extract of selected bacteria as compared to control (Figure 2(c)). Hence, this analysis showed that extracellular components of selected bacterial isolates had more antioxidant potential than their intracellular components (Figure 2).

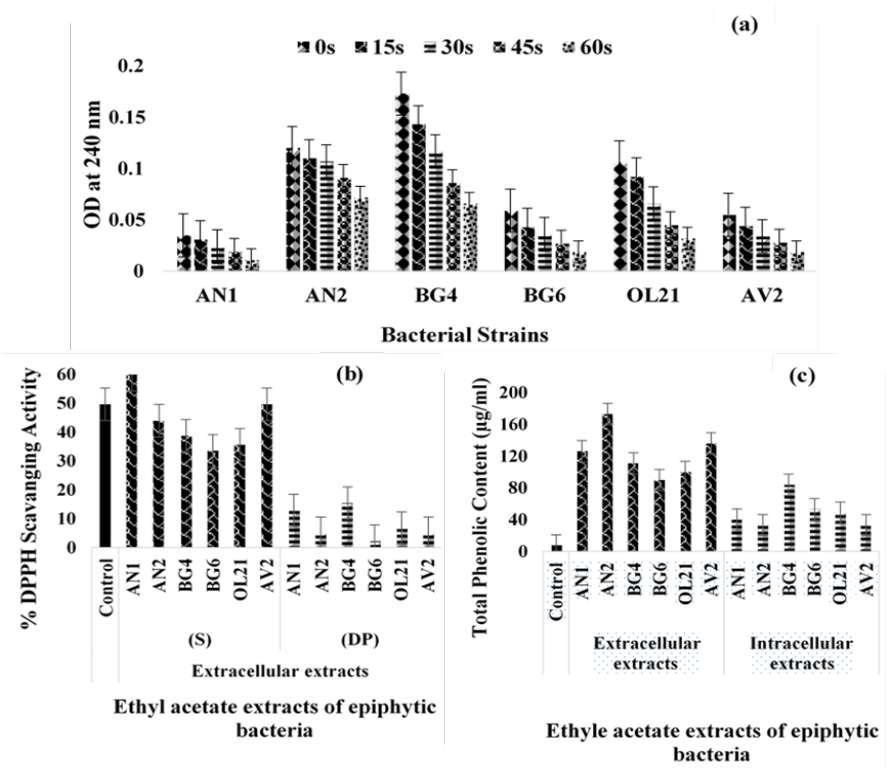

Fig. 2. Antioxidant potential of selected epiphytic bacteria (a) Catalase activity. Strain AN2, BG4 and OL21 had maximum catalase activity. (b) DPPH scavenging capability. Strong extracellular %age of DPPH scavenging capability of all selected epiphytic bacteria. (c) Total phenolic content. Extracellular TPC was significantly higher than intracellular TPC in all selected epiphytic bacteria.

3.8. Amplification of *Int1* Gene in Epiphytic Bacteria

Genomic DNA was isolated from all selected bacterial isolates for the screening of *IntI1* gene. Integrase gene *IntI1* of 280bp size was successfully detected in 66% of selected bacterial isolates (AN1, BG4, BG6 and OL21). The partial sequences of the *IntI1* gene demonstrated complete similarity with bacterial genera known to possess conserved domains of the *XerC* integrase/recombinase superfamily. These domains play roles in replication, recombination, repair, and in elements of the mobilome such as prophages and transposons (Figure 3). The sequences of the *IntI1* gene were submitted to the NCBI GenBank with the accession numbers MK882928 (for BG4) and MK882929 (for OL21).

4. DISCUSSION

Epiphytic microbial populations exhibit diversity in both plant species and the prevailing climate. In the present investigation, about 30 distinct epiphytic bacterial strains from four different medicinal plants. The selected epiphytic bacteria demonstrated resilience across a wide pH range. *Pseudomonas azotoformans* displayed optimal growth at a pH range of 5-8, while pH 7 was observed as conducive for the growth of *Paenobacillus* sp. and *Pseudomonas* sp. [21]. Epiphytic microorganisms are remarkably adaptable to environmental conditions on plant surfaces that undergo temperature fluctuations. Consequently, these bacteria may exhibit varying optimal growth temperatures. Within the scope of this investigation, it was observed that the selected epiphytic bacteria displayed robust growth across a spectrum of temperatures. *Staphylococcus* pasteuri and *Staphylococcus* haemolyticus showcased their ability to grow within broad temperature ranges, spanning from 15-45 °C and 18-46 °C, respectively. *Microbacterium paraoxydans* and *Pseudomonas azotoformans* reported their optimal growth temperatures at 28 °C and 27-31 °C, respectively [22]. Furthermore, *Paenobacillus* lactis displayed vigorous growth within the temperature range of 30-40 °C at pH 7. The selected epiphytic bacteria exhibited optimal growth in the presence of a diverse array of carbon sources, with the capacity to assimilate various carbon substrates from the plant's interior, including glucose, sucrose, and fructose.

The primary objective of this investigation was to probe into the resistance profiles of epiphytic bacteria against various heavy metals and pharmaceutical agents, considering different concentrations and exposures to environmental contaminants in air and water. Our findings unveiled the presence of six epiphytic bacterial strains

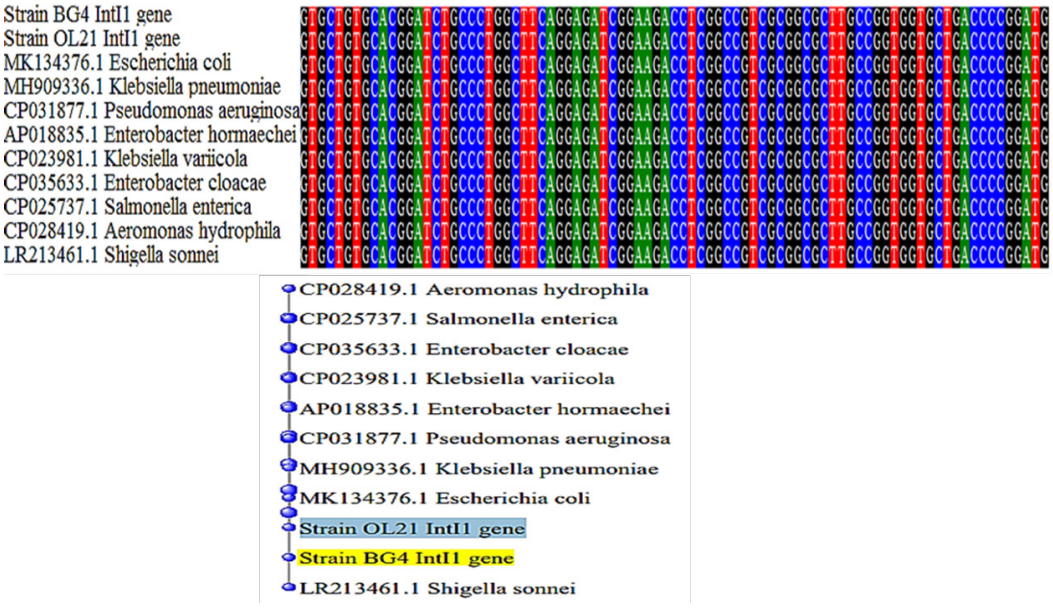

Fig. 3. Multiple alignment and of phylogenetic analysis of nucleotide sequence of *IntI1* gene indicated existence of conserved domain of *XerC* (integrase/recombinases/transposases) superfamily in multiple heavy metal and antibiotic resistant bacteria (BG4 and OL21) with 100% homology.

capable of withstanding multiple heavy metals and antibiotics simultaneously, thereby implying a potential association between resistance to heavy metals and antibiotics. To further elucidate these resistance patterns, a comprehensive analysis of 16S rRNA sequences was undertaken, leading to the identification of six epiphytic strains. These strains exhibited strikingly high similarity percentages of 99-100% with *Staphylococcus pasteuri*, *Microbacterium* paraoxydans, *Pseudomonas azotoformans, Staphylococcus haemolyticus, and Paenobacillus lactis*. Typically, *Microbacterium sp., Pseudomonas sp.,* and *Paenobacillus* sp. showed multiple metal-resistant bacteria [23]. Additionally, *Staphylococcus pasteuri* [24] and *Staphylococcus haemolyticus* [25] are known for their multiple heavy metal and drug resistance. Modification in heavy metal and antibiotic resistance, changes in membrane permeability, increased concentrations, and alterations in target sites may be the principal mechanisms involved in the development of resistance against heavy metals and antibiotics. Enhanced growth and resistance patterns detected when heavy metals like Chromium and Zinc supplemented as stressed elements to epiphytic bacteria. These epiphytic bacteria mitigate toxicity of heavy metals to plant signifying horizontal gene transfer patterns in natural environments which support plant growth under stress conditions.

This resistance pattern in epiphytic bacteria primarily arises from the release of antibiotics and heavy metals into the environment due to their extensive use [7]. Another factor contributing to this resistance pattern is the replacement of the wild-type bacteria population with a larger, resistant bacterial population on the plant surface. Recurrently multiple metal and antibiotic resistance observed in *S. haemolyticus* and *Pseudomonas azotoformans* [26].

Epiphytic bacteria have been shown to be beneficial for enhancing plant growth [27]. Both endophytic and epiphytic bacteria from medicinal plants possess the capability to produce indole-3-acetic acid (IAA), which plays a crucial role in stimulating plant cell development, division, differentiation, and gene regulation [28]. Approximately 33% of selected epiphytic bacteria produce significant amounts of IAA that are beneficial for plant growth. The production of hydrogen cyanide (HCN) is an important factor for good bacteria because it not only supports the growth of plants but also inhibits pathogenic bacteria. This attack protects the host from harmful organisms [7].

Remarkably, 83% of the chosen bacterial strains demonstrated a robust capacity for hydrogen cyanide (HCN) production, underscoring their significance as promoters of plant growth. Bacterial phosphate solubilization showcased their ability to mobilize otherwise insoluble phosphate, fostering improved plant development. Due to their epiphytic origin, only 33% of these bacteria possessed phosphate-solubilizing capabilities. A study has substantiated those endophytic bacteria resistant to heavy metals hold potential for enhancing plant growth due to their plant growth-promoting (PGP) traits [29]. Introducing epiphytic bacteria to plants offers several advantages, including heightened plant growth, disease suppression, and increased crop yields. Epiphytic bacteria also have the capacity to generate exopolysaccharides, associated with various biological functions such as antibacterial, antioxidant, biosurfactant, and immune-modulation. In our study, all selected epiphytic bacteria demonstrated the potential for exopolysaccharide (EPS) production, with a maximum EPS concentration of 77 mg/100 ml recorded. A separate report revealed that endophytic bacteria from *Withania somnifera* produced exopolysaccharides at a concentration of 0.19 mg/ml [30]. Kinetics of exopolysaccharide production is usually synchronized with stressed conditions. In this study epiphytic bacteria show resistance against heavy metals and antibiotics. These bacteria withstand the stressed conditions by producing EPS in its surroundings.

The epiphytic bacteria exhibited more potent extracellular antibacterial properties compared to their intracellular counterparts. Notably, all extracellular extracts from epiphytic bacterial supernatant displayed significant antibacterial activity against gram-positive strains, surpassing their effectiveness against gram-negative strains. This implies that epiphytic bacteria release bioactive compounds extracellularly with the ability to combat harmful pathogens [31]. Epiphytic bacteria play a crucial role in the host plant's antioxidant defense mechanism. In our study, catalase enzyme levels diminished over time in all selected epiphytic bacteria, except for *Microbacterium paraoxydans*,

while *Pseudomonas azotoformans* exhibited high catalase concentration.

In the evaluation of antioxidative activity, all ethyl acetate extracts displayed DPPH scavenging capabilities compared to the control. However, extracellular supernatant extracts exhibited 33-59% greater DPPH scavenging potential than other extracellular and intracellular extracts. The analysis of total phenolic content using the Folin-Ciocalteu assay indicated that extracellular supernatant extracts contained more total phenolic content than their intracellular counterparts. It was demonstrated that the enhanced radical scavenging ability is attributed to the specific action of phenolic compounds, acting as free radical terminators [32]. Molecular screening of resistance to heavy metals and antibiotics in epiphytic bacterial isolates revealed the presence of the Class 1 integrons/ recombinase (*IntI1*) gene. Approximately 66% of bacterial isolates tested positive for the *IntI1* gene, which exhibited 100% similarity to the conserved domains of the *XerC* superfamily (integrase/recombinase) and the alignment shows similarity with ESKAPE pathogens as well [33]. This superfamily is associated with replication, recombination, and repair, as well as the mobilome of prophages and transposons. The presence of multiple heavy metal and drug resistance is strongly correlated with integrons, containing an integrase gene and a cassette integration site where antibiotic resistance genes are integrated [30, 34]. The existence of the *IntI1* gene in the genome of these bacteria aids their survival under conditions of heavy metal exposure and antibiotic stress. In future prospects, these strains could find application in the bioremediation of heavy metals and serve as biofertilizers to promote plant growth under adverse conditions.

5. CONCLUSIONS

This study signifies the integron-mediated multiple heavy metals and antibiotic resistance pattern of epiphytic bacteria isolated from local medicinal plants. Existence of *IntI1* gene in epiphytic bacteria aids in endurance of heavy metal and antibiotic stress. Hence, further investigation at molecular level is needed to identify the further classes of integrons that supports in survival of epiphytic bacterial communities under stress environment. Moreover, these bacteria exhibited beneficial characteristics of plant growth promotion, antioxidant capabilities and EPS production making them suitable candidates to be used as biofertilizers in agriculture sector. These findings have implications for understanding the complex interactions between plants, bacteria, and environmental stressors, and may inform strategies for improving plant growth and stress tolerance in challenging environments.

6. ACKNOWLEDGEMENTS

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7. CONFLICT OF INTEREST

The authors declare no conflict of interest.

8. REFERENCES

1. R. Krishnamoorthy, S.W. Kwon, K. Kumutha, M. Senthilkumar, S. Ahmed, T. Sa, and R. Anandham. Diversity of culturable methylotrophic bacteria in different genotypes of groundnut and their potential for plant growth promotion. *3 Biotech* 8(6): 275 (2018).
2. Y. Xiong, R. Yang, X. Sun, H. Yang, and H. Chen. Effect of the epiphytic bacterium *Bacillus sp*. WPySW2 on the metabolism of *Pyropia haitanensis*. *Journal of Applied Phycology* 30(2): 1225-1237 (2018).
3. Y. Ma, M. Rajkumar, C. Zhang, and H. Freitas. Beneficial role of bacterial endophytes in heavy metal phytoremediation. *Journal of Environmental Management* 174: 14-25 (2016).
4. R.O. Schlechter, M. Miebach, and M.N.P. Remus-Emsermann. Driving factors of epiphytic bacterial communities: a review. *Journal of Advanced Research* 19: 57-65 (2019).
5. N. Wang, L.L. Stelinski, K.S. Pelz-Stelinski, J.H. Graham, and Y. Zhang. Tale of the huanglongbing disease pyramid in the context of the citrus microbiome. *Phytopathology* 107(4): 380-387 (2017).
6. J.M. Munita and C.A. Arias. Mechanisms of antibiotic resistance. *Microbiology Spectrum* 4(2): 1-37 (2016).
7. R. Karmakar, S. Bindiya, and P. Hariprasad. Convergent evolution in bacteria from multiple origins under antibiotic and heavy metal stress, and endophytic conditions of host plant. *Science of The Total Environment* 650: 858-867 (2019).

8. F. Arsène-Ploetze, O. Chiboub, D. Lièvremont, J. Farasin, K.C. Freel, S. Fouteau, and V. Barbe. Adaptation in toxic environments: comparative genomics of loci carrying antibiotic resistance genes derived from acid mine drainage waters. *Environmental Science and Pollution Research International* 25: 1470-1483 (2018).
9. G.W. Sundin and N. Wang. Antibiotic resistance in plant-pathogenic bacteria. *Annual Review of Phytopathology* 56: 161-180 (2018).
10. N. Asatiani, T. Kartvelishvili, N. Sapojnikova, M. Abuladze, L. Asanishvili, and M. Osepashvili. Effect of the simultaneous action of zinc and chromium on *Arthrobacter* spp. *Water, Air, & Soil Pollution,* 229(12): 395 (2018).
11. G. Gianluigi, L.D. Vecchio, M. Cirlini, M. Gozzi, L. Gazza, G. Galaverna, S. Potestio, and G. Visioli. Exploring the rhizosphere of perennial wheat: potential for plant growth promotion and biocontrol applications. *Scientific Reports* 14(1): 22792 (2024).
12. A. Sagar, G. Thomas, S. Rai, R. K. Mishra, and P. W. Ramteke. Enhancement of growth and yield parameters of wheat variety AAI-W6 by an organic farm isolate of plant growth promoting *Erwinia* species (KP226572). *International Journal of Agriculture, Environment and Biotechnology* 11(1): 159-171 ((2018).
13. I. Afzal, Z. K. Shinwari, S. Sikandar, and S. Shahzad. Plant beneficial endophytic bacteria: mechanisms, diversity, host range and genetic determinants. *Microbiological Research* 221: 36-49 (2019).
14. S. Bhushan, M. Gogoi, A. Bora, S. Ghosh, S. Barman, T. Biswas, M. Sudarshan, A.R. Thakur, I. Mukherjee, S.K. Dey, and S.R. Chaudhuri. Understanding bacterial biofilm stimulation using different methods-a criterion for selecting epiphytes by plants. *Microbiology and Biotechnology Letters* 47(2): 303-309 (2019).
15. J.G. Cappuccino and N. Sherman (Eds.). Microbiology - A Laboratory Manual. 4th Edition. *The Benjamin/Cummings Publishing Co. Inc. Menlo Park, California USA* (1996).
16. S.A. Medina-Salazar, M. Rodríguez-Aguilar, M.R. Vallejo-Pérez, R. Flores-Ramírez, J. Marín-Sánchez, G. Aguilar-Benítez, R. Jarquin-Gálvez, and J.P. Lara-Ávila, Biodiversity of epiphytic *Pseudomonas* strains isolated from leaves of pepper and lettuce. *Biologia* 75: 773-784 (2020).
17. S. Mustafa, S. Kabir, U. Shabbir, and R. Batool. Plant growth promoting rhizobacteria in sustainable agriculture: from theoretical to pragmatic approach. *Symbiosis* 78: 115-123 (2019).
18. S.I. Shofia, K. Jayakumar, A. Mukherjee, and N. Chandrasekaran. Study of nanoparticles impact on the growth and exopolysaccharides production of epiphytic bacteria from seaweeds. *Advanced Science Letters* 24(8): 5923-5930 (2018).
19. G. Renchinkhand, S.H. Cho, M. Urgamal, Y.W. Park, J.H. Nam, H.C. Bae, G.Y. Song, and M.S. Nam. Characterization of *Paenibacillus* sp. MBT213 isolated from raw milk and its ability to convert ginsenoside Rb1 into ginsenoside Rd from Panax ginseng. *Korean Journal for Food Science of Animal Resources* 37(5): 735-742 (2017).
20. R. Dilshad and R. Batool. Antibacterial and antioxidant potential of *Ziziphus jujube, Fagonia arabica, Mallotus phillipensis* and *Hemidesmus indicus*. *Jordan Journal of Pharmaceutical Sciences* 15(3): 413-427 (2022).
21. H.J. Kim, A.W. Lee, and C. Park. Toxicological evaluation of *Microbacterium foliorum* SYG27B-MF. *Regulatory Toxicology and Pharmacology* 100: 16-24 (2018).
22. K. Tamura, M. Nei, and S. Kumar. Prospects for inferring very large phylogenies by using the neighbor-joining method. *Proceedings of the National Academy of Sciences* 101: 11030-11035 (2004).
23. B.V. Mohite, S.H. Koli, C.P. Narkhede, S.N. Patil, and S.V. Patil. Prospective of microbial exopolysaccharide for heavy metal exclusion. *Applied Biochemistry and Biotechnology* 183: 582-600 (2017).
24. S. Alnaimat. Iron (II) and other heavy-metal tolerance in bacteria isolated from rock varnish in the arid region of Al-Jafer Basin, Jordan. *Biodiversitas* 18(3): 1250-1257 (2017).
25. R. Roychowdhury, P. Mukherjee, and M. Roy. Identification of chromium resistant bacteria from dry fly ash sample of Mejia MTPS thermal power plant, West Bengal, India. *Bulletin of Environmental Contamination and Toxicology* 96: 210-216 (2016).
26. Y. Ma, M. Rajkumar, A. Moreno, C. Zhang, and H. Freitas. Serpentine endophytic bacterium *Pseudomonas azotoformans* ASS1 accelerates phytoremediation of soil metals under drought stress. *Chemosphere* 185: 75-85 (2017).
27. A. Ambrosini and L. M. Passaglia. Plant growth–promoting bacteria (PGPB): isolation and screening of PGP activities. *Current Protocols in Plant Biology* 2(3): 190-209 (2017).
28. J.B. Yu, M. Bai, C. Wang, H. Wu, and X. Liang. Regulation of secondary metabolites accumulation

in medicinal plants by rhizospheric and endophytic microorganisms. *Medicinal Plant Biology* 3: e011 (2024).

29. M. Kaur and A. Karnwal. Screening of endophytic bacteria from stress-tolerating plants for abiotic stress tolerance and plant growth-promoting properties: identification of potential strains for bioremediation and crop enhancement. *Journal of Agriculture and Food Research* 14: 100723 (2023).
30. K.S. Singh, S. Anand, D. Aggrawal, J.K. Sharma, and V. Bahuguna. Exopolysaccharides of bacterial endophytes from medicinal plant of forest origin show antibacterial and biosurfactant properties. *The Pharma Innovation Journal* 7(4): 508-512 (2018).
31. J. Kumar, D. Singh, P. Ghosh, and A. Kumar. Endophytic and epiphytic modes of microbial interactions and benefits. In: Plant-Microbe Interactions in Agro-Ecological Perspectives. D.P. Singh, H.B. Singh and R. Prabha (Eds.) *Springer Nature Singapore* pp 227-253 (2017).
32. N.E. Es-Safi, S. Ghidouche, and P.H. Ducrot. Flavonoids: hemisynthesis, reactivity, characterization and free radical scavenging activity. *Molecules* 12(9): 2228-2258 (2007).
33. S.S. Mustafa, R. Batool, M. Kamran, H. Javed, and N. Jamil. Evaluating the role of wastewaters as reservoirs of antibiotic-resistant ESKAPEE bacteria using phenotypic and molecular methods. *Infection and Drug Resistance* 15: 5715-5728 (2022).
34. C. Mutuku, Z. Gazdag, and S. Melegh. Occurrence of antibiotics and bacterial resistance genes in wastewater: resistance mechanisms and antimicrobial resistance control approaches. *World Journal of Microbiology and Biotechnology* 38: 152 (2022).