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Endophytic Bacteria Showing Antioxidant Property from Periwinkle [*Catharanthus roseus* (L.) G. Don]

B. P. Chauhan[1], N. K. Singh[2], V. Kaswan[3], K. K. Soni[3] and N. V. Soni[4]

[1]Dept. of Plant Molecular Biology and Biotechnology, [2]Dept. of Microbiology, [4]Dept. of Genetics and Plant Breeding, C.P. College of Agriculture, S.D. Agricultural University, Sardarkrushinagar, Gujarat (385 506), India

[3]College of Basic Sciences and Humanities, S.D. Agricultural University, Sardarkrushinagar, Gujarat (385 506), India

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Corresponding bhaktichauhan016@gmail.com

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ABSTRACT

The experiment was conducted in the year, April–December, 2020 at the Department of Microbiology, C. P. College of Agriculture, Sardarkrushinagar Dantiwada Agricultural University, Sardarkrushinagar, Gujarat, India to isolate and characterize promising antioxidant producing endophytic bacteria from periwinkle plant. Twenty four endophytic bacterial cultures derived from various parts of *Catharanthus roseus* (L.) G. Don were tested for total antioxidant capacity (TAC) and total phenolics content (TPC). The isolate R1 showed highest TAC (615.46 µg AAE mg^{-1} extract) followed by the isolate R2 (308.59 µg AAE mg^{-1} extract). Correlation coefficient observed between TAC and TPC was 0.7591, which shows that phenolic compounds were greatly responsible for antioxidant capacity of the bacterial isolates. 15 isolates showing higher TAC were studied for morphological and molecular characteristics. All the 15 isolates were rod shaped and were monobacillus. Nine isolates were Gram positive whereas 6 were Gram negative. 16S *r*DNA amplification using universal primers 27F and 27R produced a band of 1.5 kb. Restriction digestion of PCR products of all the isolates with tetracutter restriction enzymes *Alu*I, *Taq*I, *Hae* III produced polymorphic diagnostic fingerprints. The dendrogram based on ARDRA profiling grouped the 15 bacteria into two groups (cluster A and cluster B) at a Jaccard's similarity co-efficient of 0.83. Cluster A contained eleven bacterial isolates whereas cluster B had only four. The isolates R1 and R2 may serve as an excellent source of antioxidants and may be exploited for commercial production of antioxidants.

KEYWORDS: 16S rDNA, antioxidant, ARDRA, endophytic bacteria, periwinkle, UPGMA

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1. INTRODUCTION

Endophytes are generally endosymbiotic microorganisms (commonly bacteria or fungi) that systematically colonize and proliferate within plant tissues without causing any sign of disease (Nair and Padmavathy, 2014). These endophytes are also capable of establishing symbiotic relationship with the plant thus making them efficient to survive and protect them from biotic and abiotic stress (Asha et al., 2023, Kumar et al., 2023, Mrugesh et al., 2022). Research reports have demonstrated the activity of bacterial endophytes against various pathogens (Atiphasaworn et al., 2017, Wang et al., 2019). Use of endophytes for production of bioactive compounds has advantage of faster production of uniform quality compounds on a large scale and the possibility of obtaining new bioactive components under different culture conditions (Numan et al., 2022, Sarjono et al., 2019). Endophytes are sometimes also responsible for the medicinal properties of the host plants (Erjaee et al., 2019, Jain et al., 2017, Rat et al., 2021). Endophytes are known to synthesize bioactive compounds that can be used by plants for defense against pathogens and some of these may be a valued drug (Fadiji and Babalola, 2020, Myo et al., 2020, Rahman et al., 2017).

Antioxidants are bioactive compounds that target free radicals in our body. Unstable atoms or molecules have free radicals due to the presence of unpaired electrons in their outer orbitals. These free radicals are very reactive and over a period of time may cause serious cell damage and even death (Sulistiyani et al., 2016, Triandriani et al., 2020). Some endophytic bacteria that show high antioxidant activity are *Pseudomonas hibiscicola, Macrococcus caseolyticus* and *Enterobacter ludwigii* (Akinsanya et al., 2015). Major antioxidant enzymes, superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx) have evolved over long period of time in living systems to play a fundamental and indispensable role in protection of most of the known biological systems from highly oxidizing free radicals (Chovanová et al., 2020, Ighodaro and Akinloye, 2018). Endophytes produce various classes of natural products and exhibit a broad range of biological activity. They are grouped into various categories as alkaloids, terpenoids, steroids, lactones, phenolic compounds, quinones and lignin (Anjum and Chandra, 2015).

Because of varied ecological niches of *C. roseus*, there is strong possibility of wide diversity in endophytic localization of bacteria inside the plant tissues. Endophytes isolated from *C. roseus* not only produce valuable therapeutic molecules but are also improve *in planta* content of terpenoid indole alkaloids like serpentine, ajmalacine, vindoline and vinblastine (Almagro et al., 2015, Anjum and Chandra, 2019, Hemmati et al., 2020, Koul et al., 2013). Endophytic bacteria *Staphylococcus sciuri* and *Micrococcus* sp. can be used as bio-inoculants to increase plant biomass and content of key terpenoid indole alkaloids within the plant, thus, providing an efficient and economic means to overcome the gap between high demand and low supply of these vinca alkaloids (Tiwari et al., 2013). Flavonol glycosides and caffeoylquinic acids are main phenolic compounds responsible for the antioxidant activity of *C. roseus* plants. *Catharanthus roseus* has compatible antioxidant activity and may be helpful for the treatment of diseases caused by free-radical oxidative stress (Nisar et al., 2017). *C. roseus* is free of side effects and cost effective and an efficient means to get rid from oxidative stress mediated diseases (Kumar et al., 2013). Endophytes can be a promising source of bioactive compounds and can be employed for production of antioxidants. These endophytes either may have intrinsic property to show higher antioxidant activity or may have acquired this property due to their close association with the host plant. Keeping in views, the objective was aimed to isolate and characterize promising antioxidant producing endophytic bacteria from periwinkle plant.

2. MATERIALS AND METHODS

Experiment for isolation and characterization of endophytic bacteria showing antioxidant property from periwinkle plants was conducted in the year, April–December, 2020 at the Department of Microbiology, C. P. College of Agriculture, Sardarkrushinagar Dantiwada Agricultural University, Sardarkrushinagar, Gujarat, India. The periwinkle plants for isolation of bacterial endophytes were obtained from the Horticultural Instructional Farm, Department of Horticulture, Sardarkrushinagar Dantiwada Agricultural University, Sardarkrushinagar.

2.1. Isolation of endophytic bacteria

The surface sterilized samples of plant parts (root, stem, leaf and petals) were cut with sterile scalpel into small pieces and were placed with the help of sterile forceps on petriplates containing nutrient agar medium. These petriplates were incubated in inverted position at 35±2°C (Cappuccino and Sherman, 2001). These petriplates were frequently checked after 48 hours for appearance of bacterial colonies on the plant parts inside petriplates. Morphologically diverse bacterial colonies were aseptically picked with the help of sterilized inoculation loop and were streaked on fresh nutrient agar petriplates. These cultures were purified by isolation of single colony and repeated sub-culturing on nutrient agar petriplates and incubating them at 35±2°C. The isolation process was repeated till monocultures were obtained. These pure cultures were maintained on nutrient agar slants and stored in refrigerator at 4°C for further experimentation.

2.2. Estimation of bacterial antioxidant capacity

The bacterial isolates were inoculated (@ 0.1 ml, OD 1.0 at 600 nm) in Erlenmeyer flasks containing nutrient broth medium (50 ml) and incubated for 38 hrs in shaking incubator at 35±2°C at 150 rpm. The bacterial isolates were then transferred into 50 ml sterile centrifuge tube and centrifuged at 5,000 rpm for 5 m. The supernatant and was separated was transferred in to separating funnel and added equal volume of ethyl acetate to it (Monowar et al., 2019). The two phases obtained were mixed vigorously for 10–15 m. The separating funnel was kept on a stand for separation of two phases from each other. Opened the cock and removed the lower layer in a beaker. Repeated the extraction two more time using the lower layer (aqueous phase). The organic solvent was collected into large pre-weighed glass plate and allowed the solvent to evaporate. The solvent being volatile in nature gets evaporated at room temperature leaving behind the crude extract of bioactive metabolites in the glass plate. After 24 h of incubation, solvent got evaporated completely. Post evaporation of organic solvent, weight of glass plate was recorded and the weight of bacterial crude extract was estimated. This bacterial crude extract was dissolved in Methanol (@ 1 mg ml^{-1}) to estimate the antioxidant capacity of the bacterial extract.

2.2.1. Total antioxidant capacity (TAC)

Total antioxidant capacity (TAC) of the bacterial isolates was measured following the phosphomolybdenum method using sulfuric acid, sodium phosphate, and ammonium molybdate. An aliquot of 0.1 ml of sample solution (crude extract) was combined in an Eppendorf tube with 1.0 ml of reagent solution (0.6 M sulfuric acid, 28 mM sodium phosphate, and 4 mM ammonium molybdate). The tube was then capped and incubated in a thermal block at 95°C for 90 m. The sample was allowed to cool to room temperature and then the absorbance of the solution of each bacterial sample was measured at 695 nm against a blank. A typical blank solution containing 1.0 ml of reagent solution and the appropriate volume of the same solvent used for the bacterial sample was incubated under the same conditions. Standard ascorbic acid was used as the control, and the Total antioxidant capacity (TAC) was expressed as µg of Ascorbic acid equivalent per mg of bacterial extract (µg AAE mg^{-1}). The TAC was calculated using the following linear equation based on the calibration curve for ascorbic acid:

$Y=0.0011X+0.0121 (R^2=0.9963)$ ……..(1)

Where, Y is absorbance at 695 nm and X is concentration of Ascorbic acid (µg ml^{-1}) (Monowar et al., 2019).

2.2.2. Total phenolics content (TPC)

Total phenolic content of the bacterial extract was estimated as per the method described by Singleton and Rossi (1965). 0.5 g of the crude extract was mixed with water and the volume was adjusted to 50 ml. It was subjected to shaking and filtration. 5ml filtrate was taken and and 1ml of FC reagent was added to it. After 6 min, 10 ml of 7% Na_2CO_3 was added. The mixture was made up to 25ml and incubated in a dark place for 90 min. OD was taken at 750nm and the phenolic content was calculated using the formula.

Total Phenolics (µg mg^{-1})=(Abs. sample×Total volume (ml)×Dilution factor)/(Slope×Sample aliquot volume (ml)× Weight of sample (g))×100 ……..(2)

2.3. PCR-based molecular characterization of the bacterial isolates

2.3.1. Isolation of genomic DNA

For DNA isolation, single colony of the endophytic bacteria (15 isolates showing higher TAC) were inoculated into freshly prepared Luria Bertani broth medium. After an incubation for 24 h, DNA isolation from the isolates were done using Thermo Scientific GeneJET Genomic DNA Purification Kit (K0721, Thermo Fisher Scientific, Waltham, US) following standard protocol.

The purity and yield of the DNA was assessed spectrophotometrically by calculating the A_{260} A_{280}^{-1}. Gel electrophoresis of genomic DNA of the bacterial isolates was carried out on 0.8% agarose gel (stained with ethidium bromide @10 mg ml^{-1}) at 65 V for 1 h in 1X tris-acetate EDTA-buffer. The gel pictures were visualized under UV-light and gel photograph was scanned using gel documentation system (UVI Tech Ltd., U.K.) to reveal the intactness of the genomic DNA. For further experimentation, the DNA concentration of all the isolates were maintained at 25 ng $µl^{-1}$.

2.3.2. PCR amplification of 16S rDNA of the bacterial isolates

Molecular identity of the isolates were deciphered by amplification of 16S *r*DNA of the bacterial endophytes using the universal primers: 27F (5'AGAGTTTGATCCTGGCTGAG-3') and 27R (5'-AAGGAGGTGATCCAGCCGCA-3'). Amplification were carried out in a 20 µl reaction volume containing 2 µl DNA, 2 µl *Taq* buffer, 1.4 µl $MgCl_2$, dNTPs 0.4 µl, Primers 2 µl, *Taq* DNA polymerase 0.4 µl and nuclease free water 11.8 µl. The reaction mixtures were then subjected to the optimized temperature conditions in polymerase chain reaction: Initial denaturation of the template DNA at 94°C for 5 m, 35 repeated cycles of denaturation at 94°C for 30 s, annealing at 55°C for 30 s and elongation at 72°C for 2 m, and a final elongation at 72°C for 10 m. The amplified 16S *r*DNA products were separated on a 1.5% (w v^{-1}) agarose gel in 1X TAE buffer and visualized by ethidium bromide staining under UV light

and gel photograph was scanned using gel documentation system (UVI Tech Ltd., UK).

2.3.3. Amplified ribosomal DNA restriction analysis

The Amplified ribosomal DNA Restriction Analysis (ARDRA) was originally developed by Vaneechoutte et al. (1993) for deciphering genetic diversity in bacteria. The technique involves digestion of 16S *r*DNA using tetra cutter restriction enzyme. In present experiment, the restriction enzymes *Msp*I, *Rsa*I, *Alu*I, *Hae*III and *Taq*I were used separately for digestion of the PCR amplified 16S *r*DNA product. The restriction mixture consisted of amplified PCR product (5.0 µl), nuclease free water (9.0 µl), 10x buffer (2 µl) and restriction enzyme (2µl). These components were gently mixed and spin down for a few second. The restriction mixture for restriction digestion was kept for incubation for 1.0 hour at 37°C. The restriction digestion fragments of the amplified 16S *r*DNA were separated by electrophoresis on 2.5% (w v^{-1}) agarose gel (stained with ethidium bromide @10 mg ml^{-1}) in 1x TAE buffer and the gel picture was scanned using five Reader gel documentation system (UV Itech, UK).

2.3.4. Data analysis of the restriction digestion product of amplified 16S rDNA

The restriction digestion profiles were used to generate binary matrix based on the presence or absence of bands. The similarity matrix was constructed following SIMQUAL program and the data were analyzed using numerical taxonomy and multivariate analysis (NTSYSpe 2.02i) software package (Rohlf, 2000). The dendrogram was based on the proximity matrix obtained from the Jaccard coefficient and Sequential Agglomerative Hierarchical Non-overlapping (SAHN) method and clustering was done using the Unweighted Pair Group Method with Arithmetic Average (UPGMA) Method (Sneath and Sokal, 1973).

3. RESULTS AND DISSCUSSION

3.1. Isolation of the endophytic bacteria

The morphologically diverse bacterial colonies were grown on the nutrient agar petriplates on different plant parts of periwinkle plant were aseptically transferred on fresh nutrient agar petriplates and purified by repeated subculturing followed by isolation of single colony. These pure cultures were maintained on nutrient agar slants (Anjum and Chandra, 2015). Twenty four bacterial endophytes were isolated in pure form from periwinkle plants and were stored in slants in refrigerator at 4°C. These isolates were named by the first letter of the plant part from which it was isolated followed by the numeral eg. R1, R2, S1, S2, L1, L2 and P1, P2 etc (Where, R=root, S=stem, L=leaves and P=petals of periwinkle plants).

Out of 24 endophytic bacterial isolates, 15 isolates that showed higher TAC were further studied in detail for their morphological and molecular characterization. All the 15 endophytic bacterial isolates were rod shaped, however they varied in their reaction to Gram's staining. Nine isolates (R1, R4, R5, R6, S1, S3, S6, P1 and P2) were Gram positive whereas six isolates (R2, R3, S2, S4, S5 and L1) were Gram negative. Thus, 60% of the isolates were Gram positive whereas 40% of the isolates were Gram negative. The cells of all the isolates appeared singly and rod shaped on the slide under microscope. Thus, all the isolates were monobacillus in nature. The length of the isolates ranged from 2.35 µm (P1) to 5.33 µm (S4) whereas cell width ranged from 1.89 µm (R1) to 4.27 µm (P2).

3.2. Bacterial antioxidant assay

These endophytic bacterial isolates were screened based on their Total antioxidant capacity (TAC). TAC was determined using the ascorbic acid standard curve. Overall, the Mean value of TAC observed was highest in the root bacterial isolates (295.18 µg AAE mg^{-1} extract) followed by the isolates from stems (159.67 µg AAE mg^{-1} extract), petals 59.22 µg AAE mg^{-1} extract, and from leaves 53.37 µg AAE mg^{-1} extract (Table 1). Among bacterial isolate from roots, the isolate R1 showed highest amount of Total antioxidant capacity (615.46 µg AAE mg^{-1} extract) followed by the isolate R2 (308.59 µg AAE mg^{-1} extract). From this table it is evident that the isolates obtained from roots possess much higher Total antioxidant capacity (TAC) as compared to the isolates derived from stems, leaves and petals.

The crude extract of bacterium *Acinetobacter baumannii* was found to exhibit TAC of 673.59±1.20 µg AAE mg^{-1} extract using Ethyl acetate as an extraction solvent (Monowar et al., 2019). Rahman et al. (2017) estimated the total antioxidant capacity in the bacterial crude extract and observed that *Stenotrophomonas maltophilia* showed highest antioxidant activity (37 µg mg^{-1} extract) among all the bacterial extracts followed by *Enterobacter cloacae*, : *Pantoea cypripedii* and *Bacillus subtilis* with 35, 34 and 31 µg mg^{-1}, respectively. The extracts of the endophytic bacteria have also been reported to exhibit reducing capacity of Mo(VI) to Mo(V). It had been observed that the antioxidant effect of endophytic bacteria was concomitant with the development of reducing agents (Juntachote and Berghofer, 2005). These reducing agents were the terminators of free radical chain reactions (Apak et al., 2016). Thus, the TAC of ethyl acetate extracts of the endophytic bacteria was regarded to be related to their reductive activity.

Mean value of TPC in the crude extracts of endophytic bacteria from roots was observed to be 42.87 µg GAE mg^{-1} extract. However, the corresponding mean values in the

Table 1: Total antioxidant capacity (TAC) and Total phenolics content (TPC) of the endophytic bacterial isolates

Plant parts	Bacterial isolates	Total antioxidant capacity (µg aae mg^{-1} extract)	Mean value of tac (µg aae mg^{-1} extract)	Total phenolics content (µg gae mg^{-1} extract)	Mean of tpc (µg gae mg^{-1} extract)
roots	R1	615.46	295.18	74.67	42.87
	R2	308.59		70.96	
	R3	294.50		40.74	
	R4	275.60		27.19	
	R5	145.70		23.26	
	R6	131.27		20.44	
stems	S1	189.00	159.67	40.59	27.05
	S2	172.85		37.59	
	S3	170.10		24.07	
	S4	159.79		24.00	
	S5	149.14		19.49	
	S6	117.18		16.59	
leaves	L1	108.59	53.37	22.15	19.46
	L2	47.42		21.70	
	L3	41.92		20.67	
	L4	41.24		19.04	
	L5	40.55		17.93	
	L6	40.55		15.29	
petals	P1	78.01	59.22	26.67	19.83
	P2	64.60		22.22	
	P3	48.80		20.07	
	P4	42.96		18.00	
	P5	40.21		16.81	
	P6	37.80		15.26	

Note: aae: ascorbic acid equivalent; gae: gallic acid equivalent; tac: total antioxidant capacity; tpc: total phenolics content

crude extracts in bacterial endophytes from stems, petals, and leaves were respectively 27.05, 19.83, and 19.46 µg GAE mg^{-1} extract (Table 1). The Total Phenolics content (TPC) was highest in the crude extract of the isolate R1 (74.67 µg GAE mg^{-1} extract) which was closely followed by the isolate R2 (70.96 µg GAE mg^{-1} extract).

Correlation between Total antioxidant capacity (TAC) and Total phenolics content (TPC) was worked out. Earlier, it had been emphasized that the phenolic compounds are one of the most important antioxidants present in the biological systems (Andreu et al., 2018, Tungmunnithum et al., 2018). However, beside phenols many other groups like flavonoids and anthocyanins are also involved in antioxidant activity of the organism (Oki et al., 2002, Andreu et al., 2018). Therefore, in this study the Total antioxidant capacity and Total phenolic content in the 24 endophytic bacterial isolates were plotted on a graph paper and correlation was calculated. We observed a strong and positive correlation between Total antioxidant capacity (TAC) and Total phenolics content (TPC) and the correlation coefficient (R^2) was calculated to be 0.7591 (Figure 1).

Sarjono et al. (2019) noted that the EC3 bacterial isolate produced highest amount of Total phenolic content (69 mg gallic acid g^{-1} sample) in a batch culture at the 32nd hour of incubation or when the bacterial growth was at the middle of the stationary phase (Sarjono et al., 2019). This value showed that in 1 gram of metabolite extract, there are phenolic compounds which are equivalent to 69 mg of gallic acid. Bacterial crude extract obtained from bacterial pure cultures isolated from *Fagonia indica* showed high range of

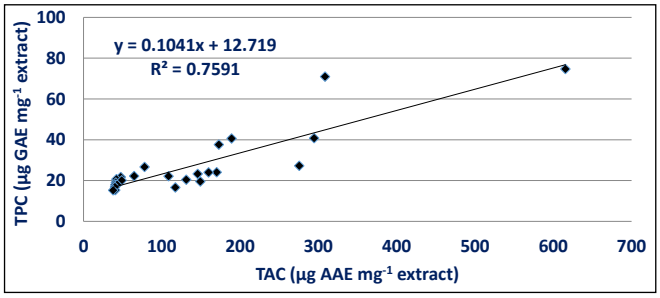

Figure 1: Correlation between Total phenolic content (TPC) and Total antioxidant capacity (TPC) in the endophytic bacterial isolates

total phenolic contents and the values varied from 13 to 243 µg gallic acid equivalents per mg of extract (Rahman et al., 2017). *Bacillus subtilis* showed high phenolic contents 243 µg mg^{-1} of gallic acid equivalents (GAE) and *Stenotrophomonas maltophilia* showed high flavonoids contents 15.9 µg mg^{-1} quercitin equivalents (QA) and total antioxidant capacity (TAC) 37.6 µg mg^{-1} of extract.

The bacteria showed that high amount of phenolic compounds production are generally regarded to have high antioxidant property (Tungmunnithum et al., 2018). Phenolic compounds as well as flavonoids were commonly known as plant secondary metabolites that hold an aromatic ring bearing at least one hydroxyl groups. More than 8000 naturally occurring phenolic compounds from plants had been reported (Ramos, 2007, Ahmed et al., 2016). According to Kinsella et al. (1993) phenolic compounds function as antioxidants because of their ability to eliminate free radicals and peroxide radicals so that they are effective in inhibiting lipid oxidation. Phenolic compounds have hydroxyl groups that could reduce radicals by donating hydrogen atoms.

3.3. Molecular characterization of the endophytic bacterial isolates

The 16S *r*DNA was highly conserved and was considered a molecular chronometer (Woese et al., 1990). This is one of the most important gene used to studied bacterial identity and evolution.

3.3.1. Isolation of genomic DNA

The isolated DNA from the 15 isolates showed higher TAC were of good quality and showed single band upon electrophoresis on 0.8% agarose gel. Moreover, the absorbance ratio of DNA (A_{260} A_{280}^{-1}) ranged from 1.70 to 1.88 with a mean value of 1.77 under spectrophotomoeter. These DNA samples were further utilized for molecular characterization through 16S *r*DNA amplification, restriction digestion of PCR amplified 16S *r*DNA and data analysis.

3.3.2. Polymerase chain reaction of the 16S rDNA

The 16S *r*DNA amplification by polymerase chain reaction with the 27F (5'AGAGTTTGATCCTGGCTGAG-3') and 27R (5'-AAGGAGGTGATCCAGCCGCA-3') revealed a band size of about 1.5 Kb on 1.5% agarose gel stained with ethidium bromide in all the bacterial isolates.

3.3.3. Amplified ribosomal DNA restriction analysis

The PCR amplified 16S *r*DNA were digested with tetra-cutter restriction enzymes, *Msp*I, *Alu*I, *Taq*I, *Hae*III and *Rsa*I, to determine whether there is a restriction fragment length polymorphism that could be used to differentiate these bacteria. The restriction digestion fragments of the amplified 16S *r*DNA were separated by electrophoresis on 3% Agarose gel stained with ethidium bromide (@ 1 mg ml^{-1}) in 1xTAE buffer and the gel pictures were scanned using gel documentation system.

The Amplified ribosomal DNA restriction analysis (ARDRA) profile of the bacterial isolates with restriction enzyme *Msp*I revealed four monomorphic fragments (650 bp, 400 bp, 250 bp, 150 bp) by each isolates whereas *Rsa*I produced monomorphic bands of sizes 520 bp, 450 bp, 390 bp and 150 bp. Thus, the restriction enzymes *Msp*I and *Rsa*I were not able to differentiate the endophytic bacterial isolates from periwinkle. However, *Alu*I produced 2 fragments of sizes 850bp and 650 bp in the isolates R1, R2, R3, R4, R5, S2, S3, S5 and P1 and one 650bp fragment in the isolates R6, S1, S4, S6, P2 and L1. Likewise, *Taq*I produced 3-5 fragments and *Hae*III produced 3 fragments in various isolates. Thus, *Alu*I, *Taq*I and *Hae*III were able to produe diagnostic fingerprints for the endophytic isolates from periwinkle (Figure 2).

The dendrogram based on the proximity matrix obtained with the Jaccard coefficient and sequential agglomerative hierarchical non-overlapping (SAHN) and clustering using the Unweighted Pair Group Method with Arithmetic Average (UPGMA) grouped these fifteen isolates of bacteria into two groups (Cluster A and Cluster B) at the Jaccard's similarity co-efficient of 0.83 (Figure 3). The dendrogram was made by combining the ARDRA profile obtained with all the restriction enzymes (*Alu* I, *Taq* I and *Hae* III) that produced polymorphic diagnostic fingerprints. The first cluster (cluster A) comprised of the eleven bacterial isolates, which was further divided in to two sub-groups. The first sub-group of the cluster A comprised of only four isolates (R1, R3, L1 and R2). However, the second subgroup of cluster A contained seven bacterial isolates S1, S2, S4, S6, S5, R5 and S3. The cluster B comprised of the only four isolates. Therefore, cluster B was narrow in range than cluster A. Cluster B was again sub-divided into two sub-groups, the first sub-group comprised of the bacterial isolates from petals P1 and P2 wereas the second sub-group of cluster B was represented by the bacterial isolates from roots (R4 and R6).

This dendrogram showed that the bacterial isolates

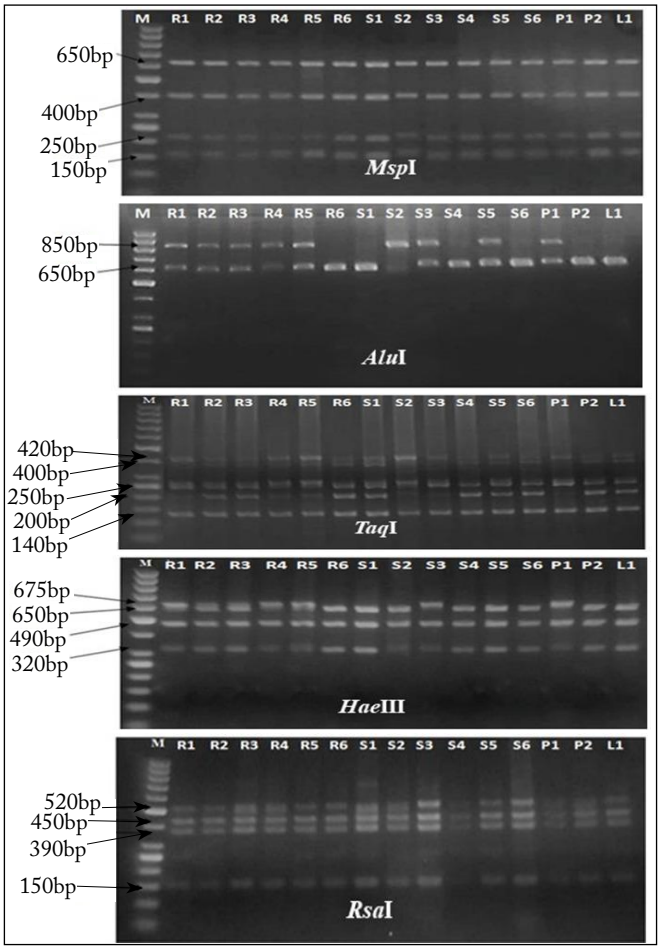

Figure 2: Amplified ribosomal DNA restriction profile of the bacterial isolates

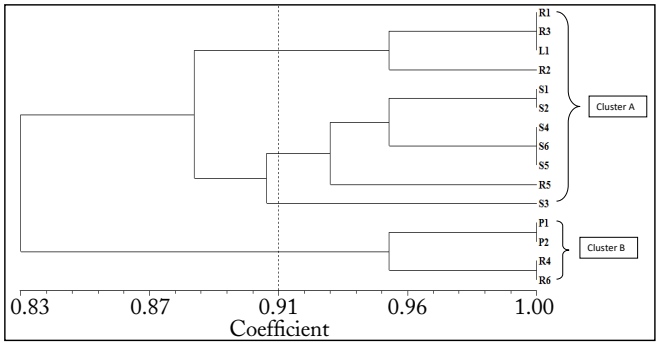

Figure 3: Dendogram based on ARDRA pattern obtained with restriction enzyme (*Alu*I, *Taq*I, *Hae*III) using Jaccard's coefficient and Unweighted Pair Group Method with Arithmetic Average (UPGMA)

obtained from roots (R1 and R3) were quite similar in their genetic composition to that of the bacterial isolate from leaf (L1). Moreover, the bacterial isolates from roots also showed similarity with the other bacterial isolates from stem, leaves and petals. This reflected that the endophytic bacteria could move from one plant part to the other and could transfer their genetic material among themselves through horizontal and vertical gene transfer.

Phylogenetic tree analysis is a method used in systematics to understand the diversity of living things through the reconstruction of kinship relations. The phylogenetic tree is used to describe the kinship consisting of a number of point and branches with only one branch connecting the two closest points. Each point represents the taxonomic units and each branch represents the relationships between units that describe the hereditary relationship with the ancestors (Amrullah et al., 2018). Hung et al. (2004) also carried out molecular characterization of selected 35 endophytic bacteria by PCR amplification of the 16S *r*RNA gene and its restriction analysis using three tetra cutters, *Hae*III, *Mbo*I and *Msp*I. Two main clusters were observed at 48% and 43% similarity coefficients in which most of the endophytes belonged. Six of the total 35 isolates (I-8, I-15, I-25, I- 68, I-121 and I-137) did not come into these clusters, showed their divergence from the rest of the isolates.

Studies had shown that the wide presence of endophytes in roots, stems, leaves, and flowers of plants (Kobayashi and Palumbo, 2000) and many bacterial genera had been isolated from plant tissues, including *Bacillus*, *Pseudomonas*, *Enterobacter*, *Aerobacter*, *Aeromonas*, *Agrobacterium*, *Chryseomonas*, *Curtobacterium*, *Erwinia*, *Flavimonas* and *Sphingomonas* (Sturz et al., 1997, Elvira-Recuenco and van Vuurde, 2000, Oehrle et al., 2000). Based on 16S rDNA sequence restriction fragment length polymorphism and 16S rDNA sequences, Chen et al. (2014) mapped 107 functional endophytic strains from ginger and grouped them into 16 genera. Although these bacteria belonged to a tremendous range of genera, *Bacillus* and *Pseudomonas* were the dominant one. 17 isolates showed antibacterial property and 90 were indole acetic acid-producing strains. Sixteen strains exhibited antimicrobial activity against *Pythium myriotylum* Drechsler, while 7 strains exhibited antimicrobial activity against *Phyllosticta zingiberi* Hori. *Pseudomonas fluorescens*, *B. megaterium*, and *Enterobacter ludwigii* produced remarkably high levels of IAA.

Methylobacterium radiotolerans MAMP 4754, isolated from the seeds of the medicinal plant *Combretum erythrophyllum,* could suppress *B. subtilis*, *B. cereus*, *E. coli*, *K. oxytoca* and *M. smegmatis* (Photolo et al., 2020). Gas chromatography mass spectrometry^{-1} (GC MS^{-1}) analysis revealed the presence of 9-octadecene, 2,4-dinitrophenyl acetate, and 2(5H)-furanone, which had been previously reported for the targeted activities. *M. radiotolerans* MAMP 4754 tested positive for antimicrobial and antioxidant activity and this was linked to the production of plant-derived secondary metabolites by this strain.

Chu and Bae (2022) are of the opinion that the bacterial endophyte communities differ in their diversity and composition, depending on the geographical location,

cultivation condition, tissue, age, and species of ginseng. They further concluded that the bacterial endophytes promote growth of ginseng through indole-3-acetic acid (IAA) and siderophore production, phosphate solubilization, N-fixation and could protect ginseng by acting as biocontrol agents. The bacterial endophytes isolated from *Panax* species had potential to produce ginsenosides and other bioactive metabolites, which are of tremendous use in food and medicine.

Bacillus siamensis HMB1 and *Bacillus aryabhattai* HMD4 cultures, derived from *Hoya multiflora* Blume plant, showed antioxidant activities. The isolate *Bacillus siamensis* HMB1 showed the highest IC50 value of 51.18 mg ml^{-1} (Pudjas et al., 2022). The antioxidant property of the endophytic bacteria associated with *Emilia sonchifolia* could be beneficial for the identification and isolation of valuable bioactive compounds (Urumbil et al., 2020). The ethyl acetate extract of six endophytic bacteria, isolated from *Emilia sonchifolia* (L.), were tested for antioxidant activity by DPPH, ferric ion reducing, nitric oxide scavenging and cupric ion reducing assays. The isolate ES1 indicated highly efficient antioxidant property. GC-MS and LC-MS analysis revealed the presence of bioactive compounds like surfactin, fengycin, iturin from the bacterial extract.

Of the six endophytes (Endo 1 to Endo 6) isolated from *Chenopodium* plants, the most active isolate Endo 2 (strain JSA11) improved the nutritive values of the treated sprouts through bioactive metabolite (antioxidants, vitamins, unsaturated fatty acid, and essential amino acids) accumulation (Almuhayawi et al., 2021). Endo 2 (strain JSA11) induced photosynthesis and the mineral uptake. Moreover, Endo 2 reduced the cyclooxygenase and lipoxygenase activity and hence increased the antibacterial activity against several pathogenic bacteria and the anti-inflammatory activities.

4. CONCLUSION

Endophytic bacteria derived from roots possessed higher TAC and TPC. Strong positive correlation (R^2=0.7591) between TAC and TPC indicated greater role of endophytic bacteria in antioxidant property of the periwinkle plant which was mainly due to the pheloic compounds produced by the bacteria. Dendrogram generated using ARDRA profile with tetracutter restriction enzymes (*Alu* I, *Taq* I and *Hae*III) indicated genetic similarity of root endophytes (R1 and R3) to that of the endophytes from leaf, stem, and petals.

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