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IDENTIFICATION OF MULTIFACETED ENDOPHYTIC BACTERIA FROM *GOSSYPIUM ARBOREUM* (CV. G.27)

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ABSTRACT

A total of 19 endophytic bacteria isolated from the root, leaf, stem and boll samples of the desi cotton *Gossypium arboreum* (cv. G.27) collected from M.C.R.S., N.A.U., Surat during 2021-22. Amongst 19 endophytic bacteria, six each were isolated from root (NAU-G27E-PR1 to NAU-G27E-PR6), leaf (NAU-G27E-PL1 to NAU-G27E-PL6) and seven from stem (NAU-G27E-PS1 to NAU-G27E-PS7). A root endophyte NAU-G27E-PR1 found to be potent with solubilization index for zinc (0.58 ± 0.015), potash (0.76 ± 0.015); IAA production (19.97 ± 0.14 ìg/ml) and gibberellic acid production (33.03 ± 0.60 ìg/ml); zone index for chitinase (0.51 ± 0.012) and protease (0.39 ± 0.010); siderophore production; antifungal activity against major soil borne pathogen *S. rolfsii* (56.64%); insecticidal activity (27.50%) against pink bollworm and belongs to highly resistant type under salt stress condition. Based on microbial and molecular characters, NAU-G27E-PR1 identified as *Bacillus halotolerance*. Exploration of endophytes with multifarious characters would be an eco-friendly approach for sustainable agriculture.

Key words : Cotton, G.27, Endophytes, PGP traits and *Bacillus halotolerance*.

Introduction

Endophytes are present in all the organisms include plant, human, animals *etc*. In plant, endophytes microbesreside within a plant tissues like root, stem and leaf supports the plant growth through acquisition of nutrients, phytohormone production; protect the plants by pathogens and insects through production of lytic enzymes and secondary metabolites (Kumala and Siswanto, 2007). Among various crops, cotton is the major crop with numerous hybrids and varieties. The four species of cotton *viz*., *Gossypium arboreum*, *G. herbaceum*, *G. hirsutum* and *G. barbadense* are the cultivated species. The first two species are diploid (2n=26) and are native to old world. They are also known as Asiatic cottons because they are grown in Asia. The last two species are tetraploid (2n=52) and are also referred to as New World Cottons. *G. arboreum* is known as desi cotton in India which is a species of cotton native to Indian subcontinent and other tropical and subtropical regions of the old world.

G. 27, a diploid cotton variety of *G. arboreum* of North Gujarat with charactersitcs of red plant body with high tannin confers tolerance to bollworms and is also more prone to biotic and abiotic stress (Xing *et al*., 2022). The endophytic bacteria within this cotton variety might possess novel endophytes that interact and useful in plant growth and protection. Additionally, they might support the plant against ecological constraints such as drought, salinity and heavy metals (Khalifa *et al*., 2015). Thus, the present study aims to isolate the endophytic bacteria within the tissues of G.27 variety of desi cotton and to study its plant growth and promoting activities.

Materials and Methods

Plant material and isolation of endophytes

Cotton plant, G.27 was collected from the Research Farm of MCRS, NAU, Surat, Gujarat, India (Latitude 23°13N; Longitude 72°41E) during 2021-22. Surface

sterilization and isolation of endophytic bacteria was done as per method of Musson *et al*. (1995).

In vitro assessment for plant growth promoting activities

Mineral solubilization like zinc, potassium and phosphate solubilization of the endophytic bacteria was done through plate assay as per standard protocols of microbiogy described by Di Simine *et al*. (1998), Parmar and Sindhu (2013) and Pikovskaya (1948), respectively. Indole acetic acid (IAA) and Gibberellic acid (GA3) production was performed as per methods of Bric *et al*. (1991) and Henderson and Graham (1962), respectively.

Detection of plant protecting traits

Microbes have inibuilt ability to produce lytic enzymes and metabolites that compete and inhibit the plant pathogens. Thus, the production of lytic enzymes *viz*., chitinase and protease was performed by spot inoculation of bacterial culture on chitin agar plate (Kuddus and Ahmed, 2013) and skim milk agar plate (Patel and Patel, 2004), respectively. Metabolites like Hydrogen cyanide (HCN) and siderophore production was determined using the method of Lorck (1948) and Schwyn and Neilands (1987), respectively.

Further, to screen the potential endophytic bacteria that possess antifungal and insecticidal activity agaist pink bollworm was also performed. Antifungal activity against the soil borne pathogenic fungi *Sclerotium rolfsii* was done using dual culture assay as per method of Li and Yang (2005) and percent inhibition was calculated using formula : [(Growth of pathogen in control - growth of pathogen with isolate)/growth of pathogen in control] × 100.

Bioassays of insecticidal activity against pink bollworm, *Pectinophora gossypiella* Saunders, was done by mass rearing of active and healthy larvae on artificial diet as per the method of Dharajothi *et al*. (2016) with minor modification for two generations. The neonate larvae emerged in third/fourth generations were utilized for bioassay studies. For the bioassay, cell suspension of each purified isolates (10^8 cells/ml) @ 1g/1ml of diet was prepared and tested with commercial preparation of *Bt* (*Bacillus thuringiensis*) powder as standard check. Ten neonates (newely emerged first instar larvae) released per replication, kept at 27°C and 65% RH for 72 and 168 hrs exposure. The corrected mortality calculated as per the Abbott's formula.

Salt tolerance ability of the isolates

It was done by spot inoculation of the bacterial culture on nutrient agar plate amended with varied concentration (100, 200, 400 and 600 mM) of NaCl, $CaCl_2$ and $MgCl_2$ in the ratio of 3:2:1, respectively (Pirhadi *et al*., 2016). Percentage reduction of growth was calculated using formula (100 x A-B/A), where A is colony diameter growth in control plate in 'mm' of the isolate and B is colony diameter growth in salt amended plate.

Microbial and molecular characterization of potent bacteria

The endophytic bacteria that exhibited maximum plant growth promoting and protecting activities were microbially and molecularly characterized. The morphological, cultural and biochemical characters of the potent endophytic bacteria was determined through gram reaction, colony characters on nutrient agar, KB003 Hi25 Identification kit and KB009 Hi-carbohydrate kit, respectively. Molecular identification was performed through 16s-rDNA sequence analysis. Bacterial genomic DNA was extracted, purified and was confirmed through agarose gel electrophoresis (Nakada *et al*., 2010). Bacterial 16S rDNA gene was amplified using primers 357F(CCTACGGGAGGCAGCAG) and 1391R (GACGGGCGGTGWGTRCA) and column purified amplicon was sequenced. Consensus sequence of 16S rDNA gene was generated and used to carry out BLAST with the database of NCBI Genbank. The evolutionary history was inferred using the Neighbor-Joining method (Saitou and Nei, 1987). The evolutionary distances were computed using the Maximum Composite Likelihood method (Nei and Kumar, 2000) and evolutionary analyses were conducted in programme MEGA7 (Kumar *et al*., 2016).

Statistical analysis

The complete randomized design (CRD) was used to study level of significance. The critical difference (CD) among the variance was calculated at $P \leq 0.05$ (Panse and Sukhatme, 1967). Results were expressed as mean with standard deviation (mean±SD) or standard error (mean±SE). The results were graphically presented using Microsoft Excel.

Results and Discussion

Isolation of endophytic bacteria

G.27, a desi cotton known for its ability to withstand biotic and abiotic stresses. The endophytic microbes associated with this plant tissues might have vast diversity along with plant growth development, protection and stress tolerance ability. Therefore, a total of 19 morphologically distinct endophytic bacterial colonies were isolated from the leaf, root and stem samples. Amongst 19 isolates; six each were isolated from root (NAU-G27E-PR1 to NAU-

G27E-PR6), leaf (NAU-G27E-PL1 to NAU-G27E-PL6) and seven from stem (NAU-G27E-PS1 to NAU-G27E-PS7) after 48-72 hrs of incubation. Earlier, McInroy and Kloepper (1994) isolated 32 bacterial genus of endophytic bacteria from the root and stem of *G. hirsutum* cultivar DES119. Bhowmik *et al*. (2002) reported 50 endophytic bacteria from the root and stem samples of *G. hirsutum* (cv. Pusa 8-6) and *G. barbadense* (cv. Suvin). In our study, the number and diversity of bacteria isolated from G.27 are less than the isolates reported by McInroy and Kloepper (1994) and Bhowmik *et al*. (2002). This might be due to the high tannin or complex biochemical features of G.27 cotton plant. Further, boll samples deprived the presence of endophytes probably due to presence of waxes, high cellulose or other biochemicals that are not suitable for bacterial growth.

Plant growth promoting traits

Qualitative assay for zinc, potassium and phosphate solubilization

Five isolates (NAU-G27E-PR1, NAU-G27E-PL1, NAU-G27E-PL2, NAU-G27E-PL4 and NAU-G27E-PL5) solubilized the zinc with SI of 0.77 ± 0.012 to0.05 ± 0.010; whereas bacteria NAU-G27E-PR1 solubilized potash with SI of 0.76 ± 0.015 at 96 hrs at 30 ± 2 °C (Table 1). Endophytic bacteria of the cotton plant especially G.27 were less studied for its plant protecting activities. However, Shahid *et al*. (2017) studied the plant growth promoting activities of rhizoshpere bacteria of cotton crop and observed that the bacterial strains coded as ARS-38 was able to solubilize zinc oxide but not the phosphate. Similarly, Hasan *et al*. (2022) found that endophytic bacteria *viz*., HNH7 and HNH9 that solubilized zinc and potash but not phosphate shown effective growth promotion in cotton plant.

Quantitative assay for IAA and GA3 production revelaed that among 19 bacteria, ten bacterial isolates produced IAA in the range of 23.08 ± 0.60 to 18.78 ± 0.26µg/ml. Bacterial isolate NAU-G27E-PS5 (23.08 ± 0.60 µg/ml) showed maximum IAA production. While single endophytic bacteria NAU-G27E-PR1 (33.03 ± 0.60 µg/ml) exhibited gibberellic acid production (Table 1). The phytohormones produced by our endophytic bacteria seems to be higher as compare to the 67 of IAA and 55 of GA_3 producing bacteria of rhizosphere of *G. hirsutum* reported by Patel and Desai (2015) that produced IAA in the range of 0.070 – 0.317 µg/ml while GA_3 in the range of 0.001- 0.364 µg/ml.

Plant protection traits

Bacterial antagonistic activities were related with the lytic enzymes and metabolites production. Qualitative assay for the chitinase showed that the isolates NAU-G27E-PR1 and NAU-G27E-PR4 produced solubilization index of 0.51 ± 0.012 and 0.25 ± 0.01 at 7th day of incubation. Further, eight bacterial isolates showed protease activity in the range of 0.76 ± 0.012 to 0.053 ± 0.006, respectively at 72 hrs incubation (Table 1). Assays for the HCN and siderophore production indicated that

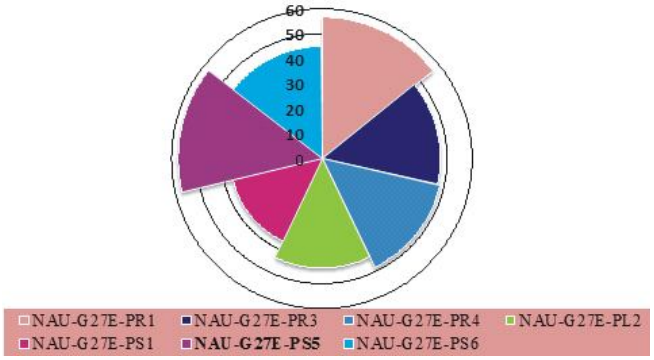

Fig. 1 : Isolates screened for percent mycelia inhibition of *S. rolfsii*.

Table 1 : Salt tolerance ability of the bacterial endophytes of G27.

Isolates	Percent (%) reduction of bacterial growth			
	100 mM	200 mM	400 mM	600 mM
NAU-G27E-PR1	-12.29*	-13.55*	-15.21*	-16.42*
NAU-G27E-PR2	10.52	25.40	30.24	50.50
NAU-G27E-PR3	-12.09*	-25.07*	-26.34*	-26.50*
NAU-G27E-PR4	5.92	10.48	21.09	27.79
NAU-G27E-PR5	9.12	13.37	35.09	45.57
NAU-G27E-PR6	-5.18*	-10.19*	-11.23*	-11.35*
NAU-G27E-PL1	9.70	27.36	50.17	68.58
NAU-G27E-PL2	11.00	16.19	35.03	35.17
NAU-G27E-PL3	6.14	11.48	25.44	27.79
NAU-G27E-PL4	23.08	30.52	42.84	46.19
NAU-G27E-PL5	12.08	15.35	17.92	18.41
NAU-G27E-PL6	12.52	28.40	40.24	50.50
NAU-G27E-PS1	-6.27*	-12.53*	-17.50*	-17.73*
NAU-G27E-PS2	12.13	34.99	46.31	66.49
NAU-G27E-PS3	16.67	34.50	36.40	50.32
NAU-G27E-PS4	6.27	17.50	24.07	32.69
NAU-G27E-PS5	23.33	34.50	36.40	50.32
NAU-G27E-PS6	7.16	18.38	34.48	50.89
NAU-G27E-PS7	20.33	34.62	46.40	65.32

* The growth of the isolates reduced with increased salt concentration but the colony size of four endophytes were larger in salt embedded plates than the normal (control) plates, might be due to osmoprtoectant or polysaccharide production by the bacteria.Hence, it gives negative values while putting in the formulaindicated that the bacteria are salt tolerant.

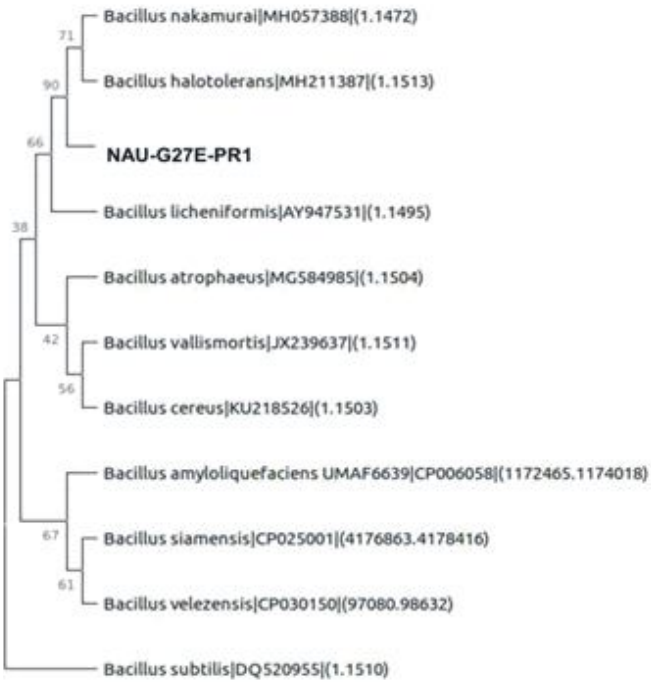

Fig. 2 : Phylogenetic tree of promising isolate, NAU-G27E-PR1.

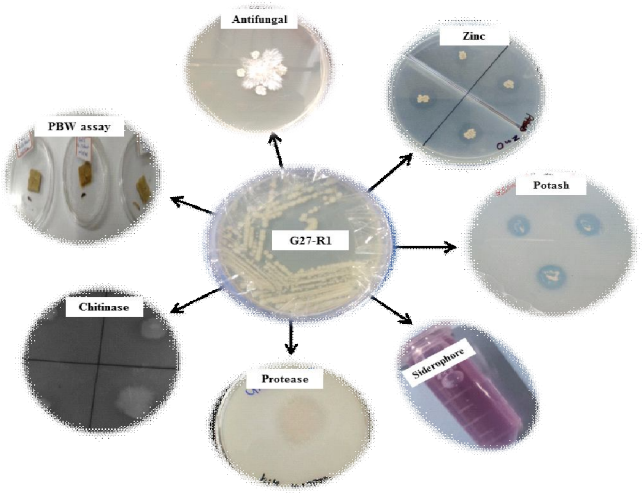

Fig. 3 : Plant growth promoting and protecting activities of NAU-G27E-PR1.

the isolates NAU-G27E-PR1, NAU-G27E-PR4 and NAU-G27E-PR2 showed orange to yellow color for the siderophore production at 76 hrs; while HCN production was not observed with any of the isolates. Bhowmik *et al.* (2002) reported proteolytic and cellulolytic activity of the endophytes *Bacillus altitudinis* HNH7 and *Bacillus velezensis* HNH9. Lytic enzymes and siderophore production of the cotton endophytes was also studied by Li *et al*. (2010) and found that among the 39 isolates, 18 showed proteolytic activity, 15 showed chitinase, while all bacteria showed siderophore production. HCN and siderophore production of cotton flora was studied by Shahid *et al*. (2017) and found that the isolate ARS-38 produced both HCN and siderophore.

Antifungal activities of the cotton endophytes was performed using dual culture assay against the major soil borne plant pathogenic fungi *S. rolfsii*. Polar graph presents the different degree of percent inhibiton of fungal mycelia (Fig. 1) ranging from 57.33 ± 0.092 to 43.72 ± 0.024 by the seven isolates at 72 hrs of incubation (Table 2). Maximum mycelium inhibition was noted with the isolate NAU-G27E-PS-5 (57.33 ± 0.092%).

Earlier, the antagonistic potential of six cotton endophytes against the fungal pathogens *Verticillium dahlia* Kleb V107 and V396 and *Fusarium oxysporum* f.sp. *vasinfectum* F108 was studied by Li *et al.* (2012) and reported effective control of the fungi. Similarly, Hasan *et al.* (2022) evaluated nine bacterial endophytes from cotton root for their antagonistic activity against *Verticillium dahlia* – 080 and found that the isolates HNH7 and HNH9 showed fungal inhibition zone 10.5 and 9.3 mm, respectively.

Insecticidal activity against Pink bollworm study revealed that amongst 19 isolates, only the isolates NAU-G27E-PR-1 and NAU-G27E-PR-5 had showed initial 27.5 ± 0.087 and 30 ± 0.010 per cent mortality of larvae of pink bollworm at 3 and 7 days after release, respectively (Table 2) against 12.50 to 40.0 per cent mortality in standard check (*Bt* powder). G.27 is the diploid cotton having red plant body and high tannin that confers tolerance to bollworms (Rajendran *et al*., 2007). Isolates, NAU-G27E-PR-1 and NAU-G27E-PR-5 showed promise as insecticidal agent.

Salt tolerance ability of the endophytes

Endophytic bacteria known to mitigate salt stress to the host plant by production of exopolysaccharide production that reduce absorption of salt by the plant. Thus, salinity tolerance of the isolated endophytes studied by incorporation of varied salts like NaCl, $CaCl_2$ and $MgCl_2$ with 100, 200, 400 and 600 mM concentration in the ratio of 3:2:1, respectively and classified for salt tolerant bacteria as per method of Pirhadi *et al*. (2016). The growth of the isolates reduced with increased salt concentration but the colony size of four endophytes (NAU-G27E-PR1, NAU-G27E-PR3, NAU-G27E-PR6, NAU-G27E-PS1) were larger than the control. It might be due to production of polysaccharide or osmoprotectants. Isolate, NAU-G27E-PL5 was found to be very resistance, 11 endophytes were resistant type while three were of moderate resistance type (Table 1).

Thus, the varied traits studied for endophytic bacteria revealed that one root endophyte (NAU-G27E-PR1) showed zinc and potash solubilization, production of GA

Table 2 : PGP traits of endophytic bacteria of G.27.

Isolates	Plant growth promoting tratis				Plant protection traits			Antifungal activity	Insecticidal activity
	Mineral solubilization (ZI)		Hormone production (ug/ml)		Lytic enzymes (ZI)		Biomolecule	% mycelium inhibition of fungi	% Larvae mortality
	Zinc	Potash	IAA	GA	Chitinase	Protease	Siderophore	*S. rolfsii*	*P. gossypiella*
Root Endophytes									
NAU-G27E-PR1	0.58 ± 0.015^{b}	0.76 ± 0.015^{a}	19.97 ± 0.14^{i}	33.03 ± 0.60^{a}	0.51 ± 0.012^{a}	0.39 ± 0.010^{c}	+	56.64 ± 0.042^{b}	27.5 ± 0.087^{b}
NAU-G27E-PR2	-	-	-	-	-	-	+	-	-
NAU-G27E-PR3	-	-	21.64 ± 0.56^{c}	-	-	0.14 ± 0.006^{g}	-	47.31 ± 0.028^{d}	-
NAU-G27E-PR4	-	-	19.98 ± 0.16^{h}	-	0.25 ± 0.01^{b}	0.20 ± 0.015^{f}	+	48.26 ± 0.032^{c}	-
NAU-G27E-PR5	-	-	-	-	-	-	-	-	30 ± 0.010^{a}
Leaf Endophytes									
NAU-G27E-PL1	0.38 ± 0.006^{c}	-	20.71 ± 0.42^{g}	-	-	-	-	-	-
NAU-G27E-PL2	0.23 ± 0.012^{d}	-	22.01 ± 0.99^{b}	-	-	0.23 ± 0.015^{e}	-	43.72 ± 0.024^{f}	-
NAU-G27E-PL4	0.77 ± 0.012^{a}	-	21.41 ± 0.51^{d}	-	-	-	-	-	-
NAU-G27E-PL5	0.05 ± 0.010^{e}	-	21.06 ± 0.19^{f}	-	-	0.76 ± 0.012^{a}	-	-	-
Stem Endophytes									
NAU-G27E-PS1	-	-	21.12 ± 0.98^{e}	-	-	0.053 ± 0.006^{h}	-	36.41 ± 0.033^{g}	-
NAU-G27E-PS5	-	-	23.08 ± 0.60^{a}	-	-	0.35 ± 0.006^{d}	-	57.33 ± 0.092^{a}	-
NAU-G27E-PS6	-	-	18.78 ± 0.26^{j}	-	-	0.57 ± 0.010^{b}	-	44.89 ± 0.081^{e}	-

and protease; three isolates (NAU-G27E-PR1, NAU-G27E-PR3 and NAU-G27E-PR4) produced IAA, protease, siderophore and showed antifungal activity; while two isolates (NAU-G27E-PR1 and NAU-G27E-PR5) showed insecticidal activity against pink bollworm. Four endophytes from leaf (NAU-G27E-PL1, NAU-G27E-PL2, NAU-G27E-PL4 and NAU-G27E-PL5) solubilized the zinc and produced IAA; two isolates (NAU-G27E-PL2 and NAU-G27E-PL5) produced protease, while one isolate (NAU-G27E-PL2) showed antifungal activity. Stem endophyte revealed that three isolates (NAU-G27E-PS1, NAU-G27E-PS6 and NAU-G27E-PS7) did show production of IAA, protease and antifungal activity (Table 2).

Identification of the potent bacteria

The endophytic bacteria NAU-G27E-PR1 was found potential based on its plant growth promoting, protecting and salt tolerance abilities as shown in Table 2. Thus, isolate, NAU-G27E-PR1 was characterized microbiologically and molecularly. Microbiogical characters showed that the bacteria was gram positive rods with short chains and produced large, irregular, entire, smooth, opaque and white colonies on nutrient agar; while biochemically it showed positive tests with nitrate reduction, methyl red, Vogus-Proskaurer's (VP), oxidase, escluline, saccharose, glucose, xylose, maltose, dextrose, galactose, sucrose, insulin and sorbitol test. Provisionaly, NAU-G27E-PR1 belongs to genus *Bacillus* based on microbial characters.

The 16S rRNA gene sequence revealed that the isolate NAU-G27E-PR1 exhibited 99.93% similarity with *Bacillus halotolerans* and the sequence was submitted in Gene bank with the accession number OQ608638.

Thus, our isolate, NAU-G27E-PR1 was identified as *Bacillus halotolerans* (Fig. 2). Recently, Thomloudi *et al*. (2021) identified an endophytic bacteria *Bacillus halotolerans* Hil4 from the leaves of medicinal plant *Hypericum hircinum* that had antifungal activity against *Botrytis*

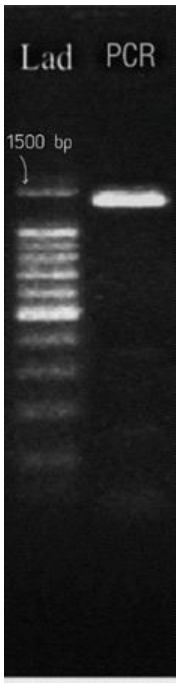

Fig. 4 : PCR amplicon of 16S rDNA (NAU-G27E-PR1) on agarose gel.

*cinerea*along with plant growth promoting and protection activities.

Conclusion

Endophytic bacteria of G.27 (*G. arboreum*) studied for its multifarious characters and observed that the endohpytes isolates from root, leaf and stem comprised of 31, 32 and 37 per cent, respectively. With regard to total PGP activities 52% of the endophytes produced IAA; 42% had protease; 36% showed antifungal activity; 26% solubilized zinc; 16% produced siderophore; 10% had chitinase and insecticidal activity; while only 5% had ability to solubilize potash and gibberellic acid production. Further, none of the endophytes showed phosphate solubilization. Salt tolerance test revealed that majority of the endophytes that reside in the root and leaf are of very resistance and resistance type while stem endophytes are of moderate resistance type. The root endophytes showed maximum activities due to richness of biochemical events of plant and microbial metabolites of rhizosphere and endopshere. Nonetheless, endophytic bacteria predominantly showed plant protection activities and salt toleranance ability than the plant growh promoting traits. Uniquely, the presence of halophilicendophytic bacteria such as *Bacillus halotolerance* long with plant protecting features inspires furhter comprehensive research regarding correlation these bacteria with biochemical constituents of desi cotton variety, G.27.

Author contributions

Conceptualization, PRP; Sampling, methodology and experimentation, Data collection and Statistical analysis, PRP, RBK, HRD, and RDP; Data analysis, interpretation and writing of draft PRP, RBK, DHP and MCP; reviewing and editing the manuscript, RBK, PRP and HRD. All authors have read and agreed to the published version of the manuscript.

Conflict of interest : The authors declare no conflict of interest.

Data availability : All generated sequences in this study are available in the Gene Bank Database under the accession number OQ608638.

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