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CONTENTS

Isolation, screening and characterization of Drought tolerant Plant Growth Promoting bacteria from Indian Himalayas

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ABSTRACT: Drought is the one of the most hazardous abiotic stress resulting in huge losses to crop yield worldwide. Plants have evolved diverse ways to eliminate the harmful effect of different stresses. The acquisition of important plant growth promoting rhizobacteria (PGPR) in rhizosphere is the most effective mechanisms acquired by plants to ameliorate different stresses in soil. The objective of the research was to isolate, screen and characterized important PGP bacterial isolates along with potential of drought stress tolerance in Poly ethyle glycol (PEG)-6000 amended medium. About 232 bacterial isolates were isolated from different crop rhizosphere and subjected to drought stress tolerance assessment. Finally, 05 isolates were screened on the basis of critical drought tolerance and PGP characteristics (Zinc solubilization, Phosphate solubilization, siderophore production) under lab condition. The selected isolates were characterized morphologicaly, biochemically and through molecular methods using 16SrDNA region. The isolates will further act as repository of PGP characteristics along with water stress tolerance which can be tested and applied in field condition for plant stress amelioration.

Key words: Characterization, drought stress PGPR, PEG-6000, rhizosphere

Abbreviations: ACC: 1 aminocyclopropane-1-carboxylate; BLAST: Basic local alignment search tool, ICAR VPKAS: Indian council of Agricultural Research- Vivekenanda Parvatiya Krishi Anusandhan Sansthan, CAS: Chrome Azurol; DNA: Deoxyribo Nucletide; HCN: Hydrogen Cyanide; IAA: Indole acetic acid, NCBI: National Center for Biotechnology Center; P: Phosphorous; PEG: Polyethylene glycol, PGPR: plant growth promoting rhizobacteria; RIDER: rhizobacteria-induced drought endurance and resilience, SDS PAGE: sodium dodecyl sulphate poly acryl amide gel electrophoresis; SI: solubilization Index, UV: Ultra Voilet; Zn: Zinc

Drought stress is one of the major agricultural problems reducing crop yield in arid and semi-arid regions of the world. Changes in mean global air temperature and rainfall patterns are leading to longer drought periods and more extremely dry years, and more severe drought conditions will affect food production in some areas around world (Lau and Lennon, 2012).

At present, strategies to improve stress tolerance in plants involve the use of water-saving irrigation, conventional breeding, and genetic engineering of drought-tolerant transgenic plants. Unfortunately, these methods are highly technical, labor-intensive, involve high cost and thus difficult to apply in practice. PGPR can be a potential option under such conditions. The bacteria that support the plant growth are known as PGPR. PGPR can be found in the plant rhizosphere in association with root systems, both at the surface and inside (endophytic associations), and which can either directly or indirectly facilitate plant growth in optimal, biotic, or abiotic stress conditions (Cassán *et al.*, 2009; Bashan and Holguin, 1998).

These microbes are free-living soil bacteria that aggressively colonize the rhizosphere and enhance the growth, and yield of plants when applied in early stages (Kumar *et al.*, 2014). The most common mechanisms involve release of metabolites which directly stimulating plant growth.

The mechanisms for plant growth promotion include: (a) production of plant growth regulators or phytohormones such as indole acetic acid (IAA), cytokinins, and gibberellins (Marques *et al.*, 2010; Glick, 1995); (b) enhancing symbiotic N_2 fixation (Sahin *et al.*, 2004; Khan, 2005); (c) solubilizing inorganic phosphate and mineralization of organic phosphate and/ or other nutrients (Jeon *et al.*, 2003); (d) antagonistic effect against phytopathogenic microorganisms through siderophore, antibiotics, enzymes, fungicidal compounds, and competition

with pathogenic microorganisms (Dey *et al.*, 2004; Lucy *et al.*, 2004).

PGPR are also linked to catabolism of molecules related to stress signalling, such as bacterial 1-aminocyclopropane-1-carboxylate (ACC) deaminase. Many PGPR have been shown to increase elthylene level in order to alleviate drought stress effects in plants (Arshad *et al.*, 2008). The drought-tolerant rhizobacteria are advantageous over others as holds promise for plant growth promotion and alleviation of plant drought stress (Zahir *et al.*, 2008).

Plants growing in the soil develop a close relationship with soil microbes residing around, on, or inside the plant roots. Certain soil microbes, colonize the root surface and inner root tissues (Gouda *et al.*, 2018), and hence plays an important role in inducing drought stress tolerance in host plants (Hartman and Tringe, 2019). Interest in the beneficial rhizobacteria associated with plants has increased recently and several studies clearly demonstrated the positive and beneficial effects of PGPR on growth and yield of different crops at different environment under variable ecological conditions (Ozturk *et al.*, 2003; Marques *et al.*, 2010; Mehnaz *et al.*, 2010; Zhang *et al.*, 2012).

Plant-growth-promoting rhizobacteria are also known for mitigation of drought stress (Vanamala *et al.*, 2021; Aslam *et al.*, 2022) in plants. PGPR alleviate drought stress via RIDER, which induces biochemical changes (Ansari and Ahmad, 2019). Various RIDER mechanisms include secretion of bacterial exopolysaccharides, phytohormone, deposition of several carbon-based components, such as sugars, amino acids, and polyamines, production of heat-shock proteins (Monteiro *et al.*, 2021).

Knowledge of the native bacterial population, their characterization, and identification is required for understanding the distribution and diversity of indigenous bacteria in the rhizosphere of specific crops (Chahboune *et al.*, 2011). With increasing awareness about the-chemical-fertilizers based agricultural practices, it is important to search for region specific microbial strains which can be used as a growth promoting/enhancing inoculum to achieve desired crop production (Deepa *et al.*, 2010). The selection of microbes with greater PGPR properties and resistance could be useful in developing drought resistance in important crop plants. With the reference of aforesaid details, the necessity to recover and characterize the microbial inoculants is more justifiable. The present study was conducted to isolate, screen and characterize the drought tolerant PGPR from different types of crop rhizosphere from rainfed areas of hills.

MATERIALS AND METHODS

Soil sampling

The soil samples were taken from Finger millet, Wheat, Soybean, Lentil and Toria crop at Hawalbagh (29° 38' 37N latitude; 79° 38' 7E longitude; 4189 feet Altitude) and Rajmash from Parsari region (29° 55' 21N latitude; 78° 59' 54E longitude; 5426 feet Altitude). Ten soil samples were taken from each plot at random location in sterile polybags with ice packs using nitrile gloves. The soil samples were taken to Agricultural chemistry and Microbiology laboratory, ICAR VPKAS, Almora for processing of rhizospheric soil and stored at 4^{o}C in refrigerated incubator before further use.

Isolation, purification and preservation of bacterial isolates

The rhizopheric soil samples (1g each) were serially diluted and spread plate on half strength Nutrient agar, Full strength Nutrient agar, Tryptic soy agar, King's B Agar, Mannitol Salt Agar for bacterial isolation employing the standard procedure of Somasegaran and Hoben, (1994). The individual colony was picked and purified on separate media using streak plate method and glycerol stocks in 50% Glycerol were made and stored at 20^{0}C for preservation.

Qualitative estimation of PGPR properties

The recovered isolates were also screened on the basis of qualitative Plant growth promoting properties. For phosphorous (P) solubilization the

active culture of bacterial isolates was prepared and spot inoculated on Pikovaskya Agar.

The plates were incubated at 32^0 C for 28 h. Halo zone formation was recorded as positive result (Mehta *et al.*, 2013). Solubilization index (SI) was calculated using the formula: SI= total diameter (colony + clear zone)/diameter of colony Similarly for Zinc (Zn) solubilization the active culture was spot inoculated on Minimal agar amended with Zinc substrates (Saravanan *et al.*, 2007) and halo zone formation after 48 hrs was the indicator of positive result. Siderophores are the chelators of iron which was estimated on Chrome Azurole Agar (CAS) through spot inoculation of active culture. The formation of orange or yellow halo was reported as positive results (Schwyn and Neilands, 1987). Vein diagram was constructed using Venny 2.1.0 online software.

Siderophore quantification

The selected bacterial isolates on the basis of qualitative results were subjected to siderophore quantification. Active culture in the rate of 1×10^8 were inoculated into sodium succinate broth and incubated for 24 hours at 120 rpm and 32^0C of temperature. The absorbance was recorded after centrifugation at 400nm and concentration was calculated using the absorption maxima and the molar extinction coefficient (e = 20,000/M/cm) according to Rachid and Bensoltane (2005).

Growth estimation in PEG 6000 amended media

The selected isolates on the basis of good PGP traits were screened for drought stress tolerance on 35% PEG amended Tryptic soy broth which is equivalent to -1.5MPa of water potential approximately and growth was observed through UV-Vis spectrophotometry and spread plate technique (Michel and Kaufmann, 1973).

Morphological and biochemical characterization of selected bacterial isolates

The selected isolates were morphologically characterized on the basis of colony morphology. For biochemical characterization gram staining, citrate utilization, skim milk agar test and iodine test were performed using the standard procedure (Aneja, 2003; Holt, 1994).

Protein assay of 10 selected isolates

After 24 h of growth the cells were pelleted and protein was extracted using cell lysis method. CellLytic B Plus working solution was prepared by mixing 11.0 ml of CellLytic B Reagent + 220µl Lysozyme solution + 110µl Protease inhibitor solution + Benzonase 550 units. Use 1.5 ml of the bacterial culture with an OD 600 of 0.5-1.0 and centrifuge the cells at 6,000 rpm for 2 minutes. Remove the spent medium and resuspend the cell pellet in 800µl of CellLytic B Plus working solution. Briefly vortex the solution to resuspend the cell pellet and mix 5 minutes to ensure full extraction of the soluble protein. Centrifuge at 10,000 rpm for 5 minutes to pellet any insoluble material. Carefully remove the soluble protein fraction from the cell debris. The isolated protein was quantified through Bradford method (Bradford, 1976) and nanodrop method. The protein samples were concentrated with chilled acetone (1:4 ratio) before any further studies. Finally, sodium dodecyl sulphate polyacrylic gel electrophoresis (SDS PAGE) analysis was done to see the diversity in protein pattern among the different soil samples (Laemmli, 1970).

Molecular Characterization of selected bacterial isolates

The screened bacterial isolates were subjected to DNA isolation through Kit (Cat No. MB505- 50PR) and 16srDNA amplification using universal bacterial primer (9bfm/1512uR) and program with initial denaturation at 96^oC for 4 min followed by 30 cycles of denaturation (96^oC for 1 min) Annealing (52^oC for 1 min) and Extension (74^oCfor 1 min) and a final extension at 74^oC for 10 min at last. The amplicon was sequenced for identification through outsourcing with the help of Himedia (Cat No. MBS104).

The sequence read were subjected to BLAST and more than 98% similarity with the database was considered for identity of the isolates. The sequence was submitted to NCBI. Evolutionary relationships of taxa The evolutionary history was inferred using the Neighbor-Joining method (Saitou and Nei,

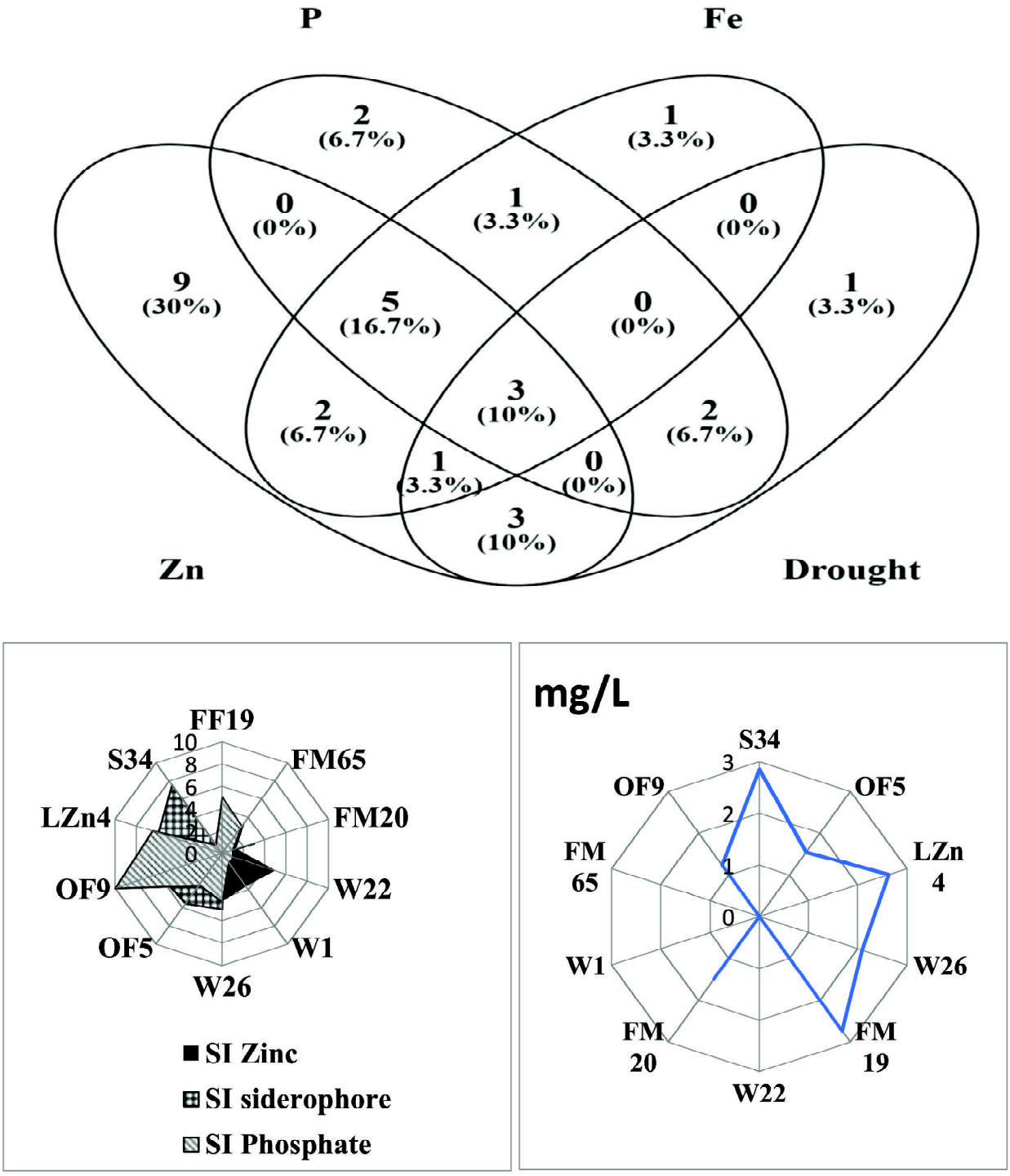

Figure 1(A): Vein Diagram to show PGPR results (Oliveros., 2007-2015); (B) solubilization index (SI) of Zinc, Phosphate and iron chelation (C) Quantification of siderophore in mg/L unit

1987). The bootstrap consensus tree from 1000 replicates (Felsenstein, 1985) is considered to represent the evolutionary history of the taxa analyzed (Felsenstein, 1985). The evolutionary distances were computed using the Maximum Composite Likelihood method (Tamura *et al.*, 2004) and are in the units of the number of base substitutions per site. This analysis involved 25 nucleotide sequences. All ambiguous positions were removed for each sequence pair (pairwise deletion option). There were a total of 1627 positions in the final dataset. Evolutionary analyses were conducted in MEGA11 (Tamura *et al.*, 2021)

RESULTS AND DISCUSSION

Isolation

A total of 232 bacterial isolates were recovered from 6 different types of rhizosphere (Finger millet, Wheat, Soybean and Lentil and Toria crop at Hawalbagh, Almora and Rajmash from Parsari Tehri Garhwal, Uttarakhand region) (Table 1).

Plant growth promotory traits

About 30 bacterial isolates were found to have good PGP properties with 23 isolates for zinc solubilization, 13 for P solubilization, 13 for siderophore production and 10 among them showed best drought stress tolerance. The relationship between different PGP traits were also illustrated with the help of Vein diagram (Figure 1(A)).

The highest phosphate solubilization was recorded in OF9 followed by LZn4 and FF19. Siderophore production was maximum in S34 followed by OF5 and W26, similarly in case of Phosphate solubilization highest P solubilization was observed in W22. The siderophore quantification was done on sodium succinate broth and highest quantity of siderophore was recorded in S34 (2.8 mg/L) followed by FM19 (2.7 mg/L), FM12 (2.6 mg/L), FM20 (1.4 mg/L) and OF5 (1.5 mg/L) whereas least siderophore was recorded in OF9 (1.2 mg/L) (Figure 1(B) and (C)).

Water stress tolerance

Only 10 isolates (FM65, W1, W22, LZn4, S34, FM20, FM19, W26, OF5, OF9) out of 133 were observed to show critical tolerance to water stress (35% PEG 6000 concentration) i.e. -1MPa. About 10% of isolates showed all the four traits including Zn and P solubilization, siderophore production and drought stress tolerance and 16.7 % of isolates showed all PGP traits except drought stress tolerance whereas about 3% of isolates were good for drought stress tolerance but did not show any PGP traits. the main objective of the study was to develop drought stress tolerant microbial inoculants to address drought stress in hilly crops so it was considered as most important traits during screening of isolates.

Characterization of bacterial isolates

The 10 bacterial isolates were characterized on the basis of morphological, biochemical characters.

Morphological characterization

The morphological features of isolates are listed in Table 2 (Figure 2).

All the isolates were gram negative with mostly rod shaped cells except W26 and A1 which were cocci.

Table 1: Details of isolation from different rhizosphere

S.No.	Crop	Number of isolates	Names of isolates
1	Finger millet	65	FM 1 to FM 66
2	Wheat	26	W1 to W26
3	Soybean and Toria inter-cropping	58	S1 to S36
			OF1 to OF15
			A1 to A7
4	Lentil	63	LP01 to LP10
			LZn1 to LZn35
			LKB1 to LKB8
			L Endo 1 to Lendo 10
5	Rajmash	20	FF1 to FF20

Table 2: Morphological characteristics of bacterial isolates

	shape	gram staining	Texture	slime producer	colony colour
FF19	rods	G-ve	non creamy	+	cream
FM65	rods	G+ve	creamy	+	cream
FM20	rods	G+ve	creamy	+	cream
W22	rods	G+ve	creamy	-	cream
W1	rods	G+ve	creamy	-	cream
W26	cocci	G-ve	creamy	-	yellow
OF5	rods	G-ve	creamy	-	brown
OF9	rods	G-ve	creamy	-	cream
LZn4	rods	G+ve	non creamy	-	white
S34	Coccobaccilus	G-ve	creamy	-	yellow

Table 3: Biochemical characteristics of selected bacterial isolates

	Starch hydrolysis	Casein hydrolysis	Simmon citrate test	Urease test	Ammonia test	methyl red	HCN	voges proskaur
FF19	+	+	-	+	+	+	-	+
FM65	-	+	-	-	+	-	+	+
FM20	-	+	-	-	+	-	+	+
W22	-	+	-	-	+	-	-	+
W1	-	+	-	-	+	-	-	-
W26	-	+	-	-	+	+	-	+
OF5	-	+	-	-	+	-	+	+
OF9	-	+	-	-	+	+	+	-
LZn4	+	+	+	-	+	-	-	+
S34	-	-	+	+	-	-	+	-

FF19, FM65 and FM20 are slime producer which is a good indicator of drought stress tolerance. The results of biochemical characteristics are shown in Table 3.

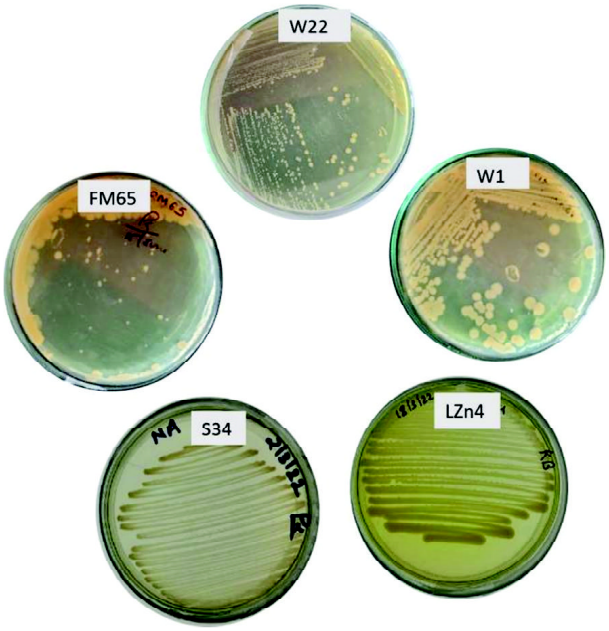

Fig. 2: Plate morphology of selected bacterial isolates

SDS PAGE analysis of selected bacterial isolates

The protein banding pattern was studied in all selected isolates. The banding pattern show the diversity among the bacterial isolates as in FF19 about 6 to 7 bands ranging from 140kDa to 15kDa were recorded. OF5 and FM65 had seven prominent bands ranging from 180kDa to 15kDa of molecular weight.

A single band of 60kDa was observed in W22. Maximum number of band were observed in of LZn4 ranging from 220 to 12 kDa approximately which were darker showing high diversity and quantity of protein. W26, S34, OF9, W1 and FM20 showed almost similar banding pattern with 7 to 8 prominent bands ranging from 180kDa to 20Kda except little dissimilarity as bands of about 220 kDa was more prominent in OF9, W1 and FM20 and 20kDa band was also observed in A1, OF9 and W1 (Figure 3).

Molecular characterization

Only 5 bacterial isolates with best properties were finally selected for molecular characterization and further studies in future. The isolates on the basis of 16sr DNA sequencing were identified and submitted to NCBI database after BLASTN. The evolutionary analysis shows the pattern of similarity among different strains. The *Presitia megaterium* strain FM65 showed least similarity with rest of the strains (Figure 4).

The plant growth promoting microorganisms not only help in growth improvement of plants but also are known to ameliorate different types of stress and drought is one of the prevalent type of stress mainly in hilly areas like Uttarakhand. The bacterial isolates recovered from rhizospheric region of different crops showed good PGP properties along with drought stress tolerance in PEG 6000 amended medium. The bacterial isolates were able to solubilize zinc and phosphorous which are one of the main micro-nutrient required for the disease suppression and growth of plants. Siderophore are the other components which help in iron acquisition and disease suppression. The beneficial effect of PGPR in maintaining adequate levels of mineral nutrients especially the P in crop production had been previously reported (Rodriguez and Fraga, 1999; Saravanan *et al.*, 2007). In the present study 232 recovered and selected isolates were also characterized on the basis of morphological, biochemical and molecular basis. Screening of isolates on every step was also carried out to obtain

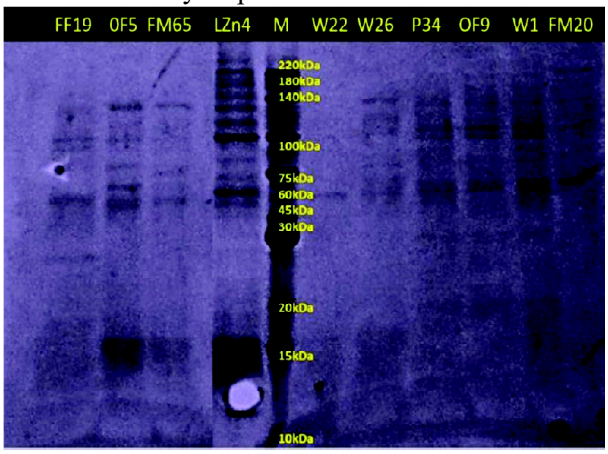

Fig. 3: SDS-PAGE analysis of selected bacterial isolates

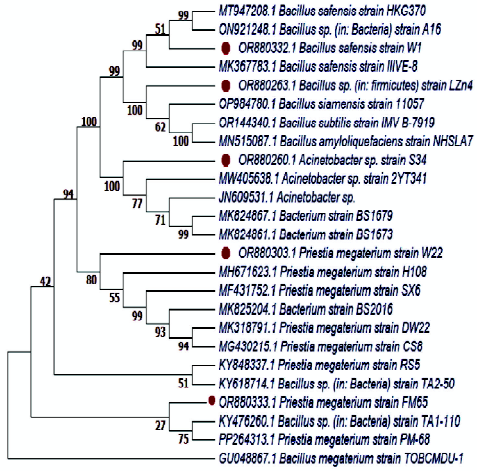

Fig. 4: Phylogenetic analysis through MEGA 11

best out of 232 isolates. Isolation and characterization of effective PGP with good traits is a very common exercise and yet very important for the exploration of different niche under different climatic condition. Similar kind of study was also conducted by Wolf *et al.* in 2001, who isolated and characterized potential rhizobacteria through polyphasic approach. They obtained two putative *Bacillus* sp. strains from rhizosphere and endophytic region. The selection of beat isolates on the basis of maximum PGP properties was done to ensure maximum benefit. According to Imran *et al.* in 2014 the inoculation of PGPR having multi-functional traits is better than having single traits. In another study isolation and characterization of PGPR was done from Himalaya region of Rawalkot, Pakistan and characterization was done on the basis of 16srDNA sequencing (Majeed *et al.*, 2015). Similarly, isolation of PGPR from high yielding Pea was done by Sherpa *et al.* (2021) which were further evaluated for plant growth promotion under pot condition. The bio-inoculants with different PGP traits have already been explored in past. The researchers have also worked out on mechanisms of different PGP activities. Phosphate solubilization by four bacterial isolates was studied by Hameeda *et al.* (2006) and Islam *et al.* (2010). The polyphasic approach for the identification of recovered isolates was applied in order to get precise results (Prakash *et al.*, 2007). As per the results of molecular characterization most of the isolates belong to genera

Bacillus which is one of the most common PGPR with spore forming potential. The spore forming ability of bio-inoculant enables its better application in field condition due to better shelf life. The five best strains (*S34-OR880260; LZn4 -OR880263; W22-OR880303; W1:OR880332; FM65-OR880333*) which were positive for all the PGP traits and water stress tolerance upto -35 Mpa can be further utilized in field for drought stress tolerance of different crops under drought situation in rainfed Himalayas.

CONCLUSION

The agriculture system in India is mainly rainfed and drought stress is one of the major problem in hilly region of Uttarakhand. The present study evaluated the potential of bacterial isolates for plant growth promotion along with drought stress tolerance. The selected isolates with best PGP and stress tolerance can be further used towards the development of effective bio-fertilizer for organic cultivation. Most of the isolates belong to *Bacillus* except S34 which belong to *Acinetobacter*, so these spore forming bacteria are also known for good shelf life in bio-formulations which is a positive indicator for their application in field conditions. These strains will be further evaluated for drought stress tolerance and other PGP traits under pot and field conditions.

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