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Screening of Rhizobacteria Isolated from Rice (*Oryza sativa* L.) and Chickpea (*Cicer arietinum* L.) from the Paddy Fields Near Mumbai and Exploring Their Potential

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***Abstract*:** Rhizosphere provides all the necessary nutrients to plants by direct and indirect means. Plant Growth Promoting Rhizobacteria (PGPR) residing in rhizosphere are able to synthesize various metabolic compounds beneficial to plants and carry out different biological processes including Nitrogen fixation and Phosphate solubilization, etc. Current study focuses on gibberellic acid (GA_3) production and Phosphate solubilization. Gibberellic acid is one of the phytohormones responsible for the growth of the plants. Phosphate solubilization property of PGPR aid in the conversion of insoluble Phosphate into more soluble form and make it available to plants. 45 different soil samples were collected from the rhizosphere of rice and chickpea plant each. 319 isolates were obtained from the soil and were screened for Phosphate solubilization and production of gibberellic acid. Solubilization indices (SI) of the isolates were calculated and 10 isolates with maximum SI were screened for quantitative approach. Similar isolates were also screened for gibberellic acid production, both qualitatively and quantitatively. Maximum activity was obtained as 1208.57 µgP/ml and 82.820 mg/ml for phosphate solubilization and gibberellic acid production respectively. Most potent rhizobacterium was identified as *Stenotrophomonas maltophilia* using Fatty Acid Methyl Esterase (FAME) analysis. Isolates were also screened for Ammonia production.

***Keywords*:** Plant Growth Promoting Rhizobacteria, Phosphate solubilization, Gibberellic acid production, FAME, Ammonia

India is one of the leading producers of agricultural crops and second largest producer of wheat and rice (Dey et al. 2020). Agricultural microbiology is a present principal research field responsible for the transfer of knowledge from general microbiology and microbial ecology to agricultural biotechnologies (Wang et al. 2009). Decrease in crop productivity and depletion of nutrients are the main drawbacks of intensive cultivation. Chemical fertilizers have depleted the natural resources and soil fertility. Thus, biofertilizers have become an important source to overcome these problems. Biofertilizers can be categorized as an organic product consist of a specific microorganism in concentrated form derived from rhizosphere or interior regions of the plant (Mishra 2014). Plants exhibit complex network of interactions with the soil microorganisms. Plant growth promoting rhizobacteria (PGPR) possess certain characteristics which directly or indirectly affect the growth of the plants (Arunjith and Sheeba 2021). Thus, soil bacteria play the key role in biogeochemical cycles and have been used for crop production for decades. The direct and indirect mechanisms of PGPR include nitrogen fixation, phosphate solubilization, auxin production, ammonia production, etc. Diverse group of microorganisms have been reported to solubilize insoluble phosphorous complexes and make it available in more usable form (Jha and Saraf 2015). Organic Phosphorous solubilization is also termed as mineralization of Phosphorous. This process arises in soil because of plant and animal remains, which happen to be the source of large amounts of organic Phosphorous compounds (Rodriguez and Fraga 1999). Gibberellins (GA_3) are prevalent phytohormones that elicit multiple metabolic functions requisite for plant growth. Such include flowering, fruit ripening and senescence, etc (Kim et al 2009). Bacteria such as *Azotobacter*, *Bacillus*, *Serratia*, *Pseudomonas*, *Enterobacter* have been shown to posses the abilities of Gibberellin production and Phosphate solubilization (Ahemad and Kibret 2014, Ambawade and Pathade 2015).

Present study deals with the isolation and characterization of microorganisms from the rhizosphere of Rice and Chickpea. Being seasonal crops, soil sampling was done in different months, different climates. Potent isolates were checked for their phosphate solubilization activity and gibberellic acid production activity. Microorganisms, since being rhizobacteria, happen to be a good biofertilizers option and can overcome the problems associated with agricultural aspects such as chemical fertilizers, soil infertility and minimum crop production.

MATERIAL AND METHODS

Sampling: Rhizosphere soil samples of rice (*Oryza sativa* L.)

and chickpea (*Cicer arietinum* L.) were collected from different regions of paddy fields of Karjat, Maharashtra, India. Approximately 0.5 to 1cm of the surface soil was scrapped off by means of spatula to avoid the contamination by surface microflora. A total of 45 samples each were collected from the rhizospheres of both rice and chickpea. Samples were brought to the laboratory in a zip lock bag and stored at 4°C until further processing. No particular sterility is maintained while sampling because of less chance of inclusion of air microflora (Reetha et al 2014).

Enrichment and isolation: One gm of each soil sample was inoculated in 40 ml of sterile Nutrient broth and incubated at room temperature for 48 hours on shaker. After incubation, loopful from each enriched broth was streaked onto sterile Nutrient agar plates. Plates were incubated for 48 hours at 28°C and the isolated colonies were further purified and maintained on Nutrient agar (Bharucha et al 2013).

Qualitative screening for phosphate solubilization: Purified isolates were spot inoculated on sterile Pikovaskaya's agar medium. Plates were incubated for 48 hours at room temperature. Plates were observed for the zone of clearance around the colonies (Suman et al.2016).

Qualitative screening for gibberellic acid (GA_3) production: GA_3 production was assayed qualitatively by phosphomolybdic acid method (Graham and Henderson 1960). Isolates giving positive results on Pikovaskaya's plates were grown in sterile Nutrient broth with Tryptophan and kept for incubation at room temperature for 48 hours. Cell free supernatant was obtained by centrifugation of enriched broth at 10000 rpm for 10 minutes. 10ml of supernatant was mixed with 5 ml of Phosphomolybdic acid reagent (12gm Phosphomolybdic acid in 250ml Ethanol). Mixture was boiled in a boiling water bath for 1 hour and tubes were observed for green coloration.

Quantitative screening for phosphate solubilization: Isolates giving clear halo around the colonies on Pikovaskaya's medium were checked further for quantitative study. Positive isolates were enriched in 40ml sterile Pikovaskaya's broth on shaker for aeration. After 48 hours of incubation at room temperature, cell free supernatant was obtained by centrifugation of enriched broth at 10000 rpm for 10 minutes. It was then subjected to Molybdenum blue method for quantification. 10ml of 1:10 diluted supernatant was mixed with 20ml Ammonium Molybdate solution and 0.25 ml of 2.5% Stannous Chloride. Absorbance of color developed was measured spectrophotometrically at 660nm and concentration was determined using standard Phosphate graph (Wei et al 2017).

Quantitative screening for gibberellic acid (GA_3) production: Positive isolates were grown in sterile nutrient broth with tryptophan. Samples were kept for incubation at room temperature for 48 hours in shaking condition. Cell free supernatant was obtained by centrifugation of enriched broth at 10000 rpm for 10 minutes. 10ml of cell free supernatant was mixed with 5ml of phosphomolybdic acid reagent. Mixture was then placed in boiling water broth for 1 hour. After boiling, tubes were cooled in an ice water bath. Absorbance was measured spectrophotometrically at 660 nm and concentration was determined by standard Gibberellic acid graph (Graham and Henderson 1960).

Ammonia production: Isolates were grown in sterile Peptone water broth and kept for incubation for 24 hours at room temperature. Nesseler's reagent was added to the tubes after incubation and checked for deep orange yellow colouration (Shobha and Kumudini 2012).

Identification of potent bacterial isolate: Most potent bacterium was analyzed for the identification by fatty acid methyl esterase (FAME) analysis using MIDI Sherlock microbial identification system. Isolates were analyzed with gas chromatography method and isolates were identified by their fatty acid composition. Procedure was followed as per protocol. Cells were harvested and are placed in a clean test tube. This follows the method of saponification where reagent mixture containing Sodium Hydroxide, Methanol and distilled water is added to the tubes containing cells. Tubes are vortexed and put in a water bath for 30 minutes and cooled further. After saponification, methylation process involves the addition of reagent 2 which is a mixture of 6N HCl and Methyl Alcohol. Tubes are again heated and further cooled. Extraction process involves the addition of reagent 3 (Hexane and tert-butyl ether) which extract the fatty acid methyl ester into the organic phase to be used further for gas chromatography. Before chromatography analysis, samples are washed using Sodium Hydroxide and distilled water. Samples were subjected for Gas chromatography analysis of fatty acid methyl esterase.

RESULTS AND DISCUSSION

Enrichment and isolation: After enrichment of soil samples, total of 319 different isolates were obtained on Nutrient agar. Isolates were purified further and stored on Nutrient agar slants at 4°C.

Qualitative screening for phosphate solubilization: Out of 319, 58 isolates showed clear zone on Pikovaskaya's agar after incubation. Solubilization index (SI) of each isolate was calculated (Table 1). Final 30 microorganisms showing maximum SI were processed further for quantitative study. Clear halo zone by the isolate was obtained (Fig. 1).

Qualitative screening for gibberellic acid (GA_3) production: Thirty isolates giving Phosphate solubilization

index above 1.50 also showed positive results for Gibberellic acid production. Green coloration was observed in the tubes after boiling the mixture in the water bath.

Quantitative screening for phosphate solubilization: All 30 isolates were subjected for quantitative study by molybdenum blue method and the 10 isolates showing maximum solubilization activities were selected for future study (Table 2).

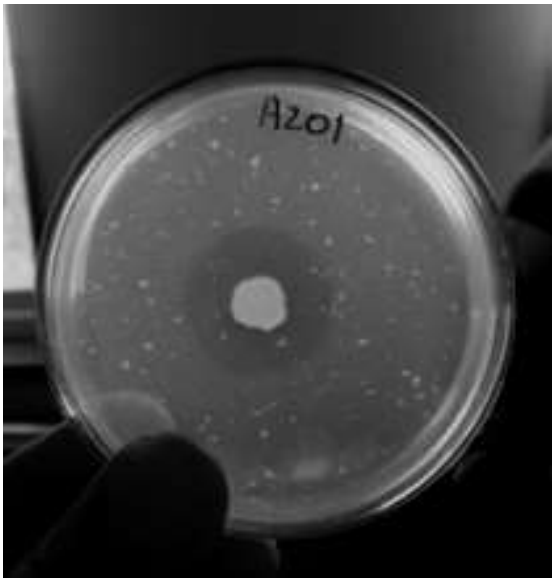

Fig. 1. Organism showing clear zone around the colony

Quantitative screening for gibberellic acid (GA_3) production: All the 30 isolates were subjected for Gibberellic acid quantification by Phosphomolybdic acid method. Above potent isolates showed similar results for the GA_3 production described in Table 2.

Ammonia Production

Above potent isolates which were incubated in Peptone water developed deep yellow-orange coloration after addition of Nesseler's reagent indicating Ammonia production (Table 3).

Identification of isolate: The most potent isolate BDC23 was identified as *Stenotrophomonas maltophilia* on the basis of fatty acid composition. Gas chromatogram of the isolate and dendrogram is given below in Figure 4 and 5 respectively. Distribution of the data set and histogram was displayed in Figure 6. Figure 7 depicts the rooted NJ tree which shows distance of isolate from related organisms.

Phosphate solubilizing bacteria (PSB) as inoculants increases the Phosphorous uptake by the plant roots. Amount of solubilized Phosphorous was 1208.537 ± 34.880 µgP/ml for isolate BDC23. These results agree with the values by Liu et al (2014). Their study obtained the results of phosphorous solubilization as 717 ±12.7 µgP/ml from the

Table 1. Solubilization indices of the isolates

Isolate	Solubilization index	Isolate	Solubilization index	Isolate	Solubilization index
BAC 11	2.09	BDC 31	3.62	SAC 31	2.05
BAC 12	1.62	BDR 22	1.33	SAR 21	2.20
BAC 13	1.01	BDR 31	2.09	SAR 22	1.32
BAC 21	1.13	BDR 32	1.41	SBC 12	1.95
BAC 22	1.53	MAC 11	1.62	SBC 31	1.44
BAR 11	1.99	MAC 12	2.04	SBC 32	1.55
BAR 13	1.25	MAC 13	1.05	SBR 11	1.10
BAR 21	1.56	MAC 22	3.55	SDC 11	3.42
BAR 22	1.33	MAR031	2.33	SDC 13	2.04
BAR 32	1.55	MAR 33	1.19	SDC 21	2.11
BBC 12	2.75	MBC 12	1.77	SDC 22	1.03
BBC 13	1.81	MBC 13	1.13	SDC 23	1.39
BBC 21	1.09	MBC 31	1.26	SDR 12	1.54
BBC 22	1.83	MBC 32	1.82	SDR 13	1.95
BBC 31	1.22	MBR 21	1.31	SDR 21	1.36
BBC 32	1.75	MDC 11	2.09	SDR 23	1.74
BDC 12	1.47	MDC 12	1.19	SDR 32	2.21
BDC 13	1.84	MDC 31	2.50	SDR 33	1.11
BDC 14	1.12	SAC 21	2.33		
BDC 23	3.71	SAC 23	1.12		

microorganisms obtained from *Areca catechu* L. (Betel nut). Experiment conducted by Zamoum et al (2015) demonstrated the P solubilization activity of 702 mg/L from the isolate ZL2 which was isolated from native plants of Algerian Sahara. Goudjal et al (2016) agrees with the method and the results obtained from our findings. Values described in table 1 for isolate BDC23 and other isolates were found to be much higher than those obtained by Perez et al (2007) and Pradhan and Shukla (2005). Gibberellins (GA_3) are important plant regulators which are concerned with the regulation of plant responses to external environment (Shah et al 2007).

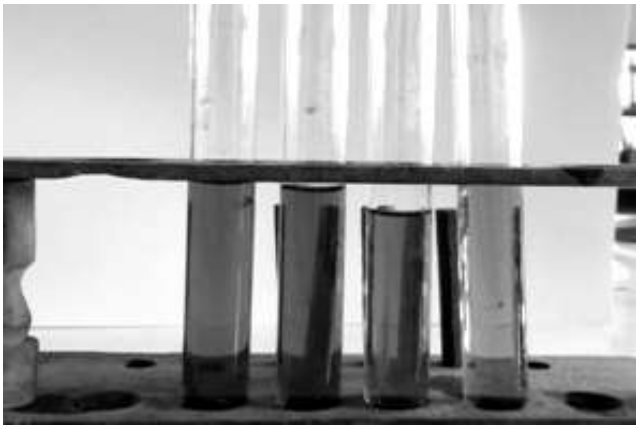

Fig. 2. Green coloration for GA_3 production

Table 2. Phosphate solubilization and Gibberellic acid production by potent isolates(Mean ± standard Deviation

Isolate	Phosphate solubilization (µgP/ml)	Gibberellic acid production (µg/ml)
BBC 12	508.883 ± 16.679	58.563 ± 4.016
BDC 23	1208.573 ± 34.880	82.820 ± 2.316
MDC 31	615.757 ± 9.764	87.052 ±1.905
SDC 11	485.093 ± 23.424	72.709 ± 1.240
BDC 31	740.470 ± 23.480	48.594 ± 0.739
SAC 21	804.187 ±12.163	78.076 ± 1.293
MAR 31	786.007 ± 33.741	49.402 ± 1.337
MAC 22	495.800 ± 41.764	69.132 ± 2.737
SAR 21	1194.980 ± 15.301	80.486 ± 1.251
SDC 21	800.450 ± 17.182	54.440 ± 1.902

Table 3. Production of ammonia by potent microbial isolates

Isolate	Result
BBC 12	+
BDC 23	+
MDC 31	+
SDC 11	+
BDC 31	-
SAC 21	+
MAR 31	-
MAC 22	+
SAR 21	-
SDC 21	-

Key: + = Ammonia production; - = No production

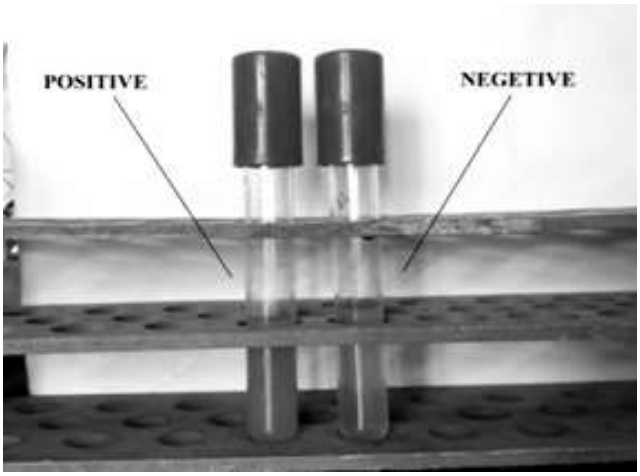

Fig. 3. Ammonia production

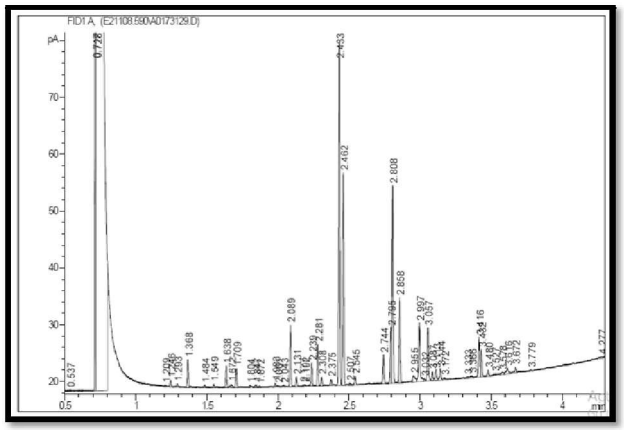

Fig. 4. Gas chromatographic run of BDC23

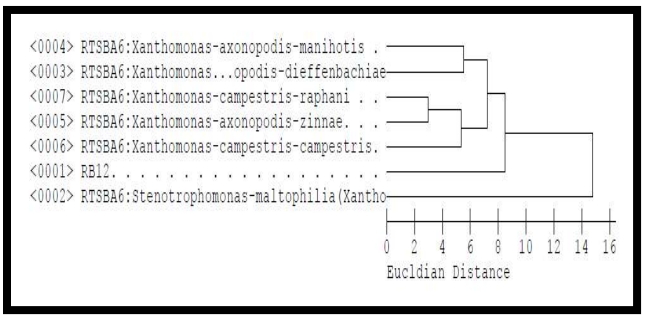

Fig. 5. Dendrogram (Pair matching based on fatty acid composition)

Fig. 6. Histogram (Distribution of the data set and graphical summary)

Index	Sel	Volume:Filename#Cntr	Bottle	ID Num	Sample ID
1	Y	DATA:E211085.90A#17	15	3129	RB12
2	Y	RTSBA6 # 802		802	Stenotrophomonas-maltophilia(Xanthomonas, Pseudomonas)
3	Y	RTSBA6 # 878		878	Xanthomonas-axonopodis-dieffenbachiae
4	Y	RTSBA6 # 882		882	Xanthomonas-axonopodis-manihotis
5	Y	RTSBA6 # 891		891	Xanthomonas-axonopodis-zinnae
6	Y	RTSBA6 # 893		893	Xanthomonas-campestris-campestris
7	Y	RTSBA6 # 894		894	Xanthomonas-campestris-raphani

Gibberellins production by microbial inoculants increases the seed germination, floral induction, fruiting and various other regulations, etc. (Bottini et al 2004). Isolates MDC31 and BDC23 produced Gibberellic acid as 87.052 ± µg/ml and 82.820 ± µg/ml respectively. Pandya and Desai (2014) Gibberellic acid production which was in the range of 7.5 mg/L to 93.93 mg/L. Sivasakthi et al (2013) observed that isolate *Pseudomonas fluorescens* L. reported maximum Gibberellic acid production of 5.96µg/ml and least production (2.89µg/ml) were obtained by *Bacilus subtilis* L.

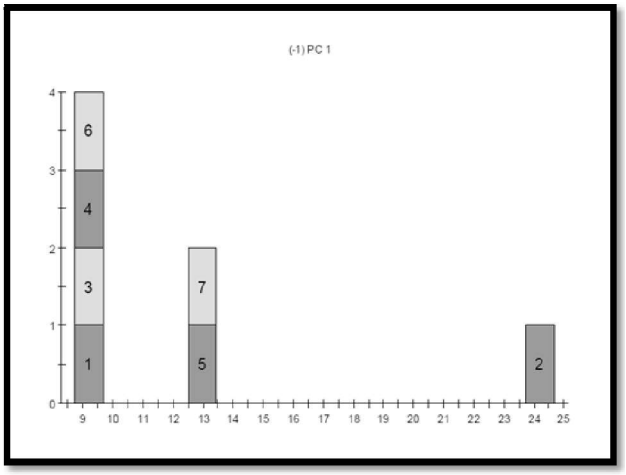

Fig. 6. Histogram (Distribution of the data set and graphical summary)

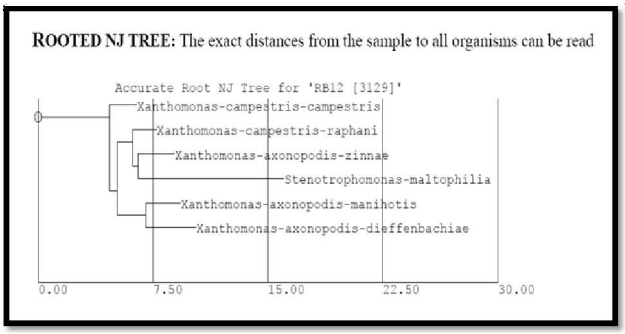

Fig. 7. Rooted NJ Tree to display the exact distance of sample from all related organisms

Out of 10 isolates, 6 microorganisms were positive for Ammonia production. Ammonia production increased the plant biomass significantly by *Bacillus subtilis* MA-2 and *Pseudomonas fluorescence* MA-4 (Mishra et al 2010). Study carried out by Singh et al. (2020) states that out of 56 rhizobacterial strains, 16 were found positive for Ammonia production. Results obtained by Suman et al. (2016) wherein isolate PLP and DMP 1 were able to produce significant amount of Ammonia (Suman et al. 2016). Despite being an opportunistic human pathogen, *Stenotrophomonas maltophilia* has been found beneficial for plant interactions. Messiha et al. isolated bacterial samples from the rhizosphere of Eggplant and after FAME analysis, potent isolate was identified as *S. maltophilia* (Messiha et al 2007).

CONCLUSION

Present investigation focuses on the bacteria which are capable for producing phytohormones such as Gibberellic acid and other Auxins along with Phosphate solubilization activity and could be easily isolated and further exploited for agricultural use. Synthetic fertilizers degrade the quality of soil, pollute surface and groundwater, and exert harmful effects on soil microflora. Thus, microbial applications, so called biofertilizers, have been proven to be the best remedy towards these problems. Microbial communities carry out essential part of nutrient recycling, pathogen suppression, etc. Isolates obtained in this study can be used as potential biofertilizers and can increase the crop productivity over mineral fertilizers.

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