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Research Article

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Biochemical and molecular characterization of grape rhizospheric microorganisms in Vani and Nandur Naka regions of Maharashtra, India

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ABSTRACT

Grape rhizosheric soil (Nasik region) harbors vast microbial abundance and diversity. In the current study we report two species of bacteria and fungi and grown on various medium like PDB, King's B, Jensen, SDA. Further biochemical, morphological and molecular studies on specific strains were carried out. Soil carbon, Electrical conductivity and pH were also estimated. The bacterial species are plant growth promoting microorganisms (PGMS) which are screened for their beneficial traits like Indole (IAA) production and Urease production capabilities. Species of pseudomonas and A. Niger are potentially responsible for P solublization, Azotobacter and Azotobacter produces IAA and GA which are plant growth promoters. Aspergillus spp. suppressed the growth of anthracnose and the fluorescent Pseudomonads shows the maximum inhibition zone for pathogen. The use of these chemical components as well as microbial reactions help in nutrient availability, growth and development of plants which results in more crop production, and they can be improved by manipulating the beneficial rhizosphere microorganisms.

Key words: Grape Rhizosphere, Bacteria, Fungi, Molecular Identification,

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INTRODUCTION

Numerous of rhizosphere microorganisms recover soil quality, enhance crop production and protection, conserve natural resources and eventually create more sustainable agricultural production and safe environment. Grape is one of the most remunerative horticulture crops of tropical India [1,2]. Maharashtra stands number one is area and production of grape with an area of 9,721 ha producing 1, 67,000 tons of grapes with a productivity of 17.2 t/ha. Maharashtra ranks first in terms of production accounting for more than 75% of total production and highest productivity in the country. The total area under grape in India is estimated to be 57,800 ha with an annual production of 14, 72,800 tones. Major grape growing districts of Maharashtra are Nasik, Satara, Sangli, Ahemad Nagar, Pune, Latur, Solapur and Osmanabad, Nashik stands first among all these districts [3].

In India more than 20 varieties of grapes are civilized. However only a dozen are commercially grown, like Sharad seedless, Sonaka, Tas-A-Ganesh, Flame seedless, Thomson, Nanasaheb purple, Jumbo, Chennin Blanc, Dilkush, Sauvignon Blanc, and Shirazet. Thomson, Tas-A-Ganesh, Sonaka. From previous studies it has been documented

that rhizosphere bacterial abunance can reach between $10^{10}$and 10^{12} cell per gram of soil [4]. The plant growth promoting rhizo microorganisms have bio control potentiality in grouping with N_2 setting and P- solubilizing organisms so they can play a pivotal role in initiating easily rooting and seedling growth of rootstock plants. Plant growth promoting Rhizobacteria (PGPR) can be an assist for sustaining agriculture and can reduce the negative impact of inorganic pesticide and fertilizers. Rhizosphere samples were collected from the grape field located in Vani and Nandur Naka Region of Nasik District and microorganisms were isolated and screened from rhizosphere soil. The current study is to isolate and molecularly characterize bacteria and fungus from grape rhizosphere.

EXPERIMENTAL SECTION

Collection and Analysis of rhizospheric soil

Rhizosphere soil was collected from grape field located at Vani and Nandur Naka regions (20° 02' N 73° 50' E) for isolation of microorganisms. The soil pH was measured by using electrometric meter the ideal pH range for availability of nutrients is 6.5 to 7.5 after that Electrical Conductivity also estimated based upon the principle that conductivity of soil is nearly proportional to the salts concentration for that EC meter was used. Soil organic carbon has been determined based on the Walkley-Black chromic acid wet oxidation method [5].

Isolation of Grape Rhizobacteria, Phosphate solubilizer from Soil Samples

The bacteria *Azotobacter and Pseudomonas* were isolated from grape rhizosphere soil samples and refined by using serial dilution method [6] and plated on Jensen and Kings B agar media. Then plates were incubated for 7 days at 37 °C. Small water droplets like glistening and Yellowish small sparkling colonies were appeared on the plates after that this culture was purified by repetitive streaking. The purified isolates were transferred to the slants of the similar medium and stored for further studies. All the samples grown on Pikovskaya's agar medium [7] and incubated at 30°C for 9 days and colonies with strong zones were purified, sub cultured and maintained on the slants of Pikovskaya's agar for further use.

Identification of P-solublizers and GA producers

In the biochemical study Gram staining, IMViC Test, Urease Test, Catalase Test and Motility Test were performed after Gelatin liquefaction [8]. Starch hydrolysis, Casein hydrolysis, Hydrogen sulfide production and Acid methods were carried out also qualitative test for IAA and GA production carried out [9,10]. Phosphate solubilization was determined according to Arun Kumar et al [11].

Isolation of Fungi from soil sample

The same protocol (above mentioned) was followed for isolation of fungi, instead of media the Potato dextrose Agar for *Trichoderma spp* and Sabouraud Dextrose Agar for *Aspergillus spp* was used with 10 day incubation period at 37°C. The samples were identified up to basic level grounded on their colony and spore morphology and microscopic examination as charted in the Bergey's Manual [8].

Total genomic DNA isolation from rhizospheric bacteria

Genomic DNA isolation was carried out by CTAB method [12] Bacteria from drenched liquid culture are lysed and proteins removed by digestion with Proteinase K. Cell wall debris, polysaccharides and remaining proteins are removed by selective precipitation with CTAB. The high molecular weight DNA is recovered from the resulting supernatant by Isopropanol precipitation. Bacteria from saturated liquid culture are lysed and proteins removed by digestion with Proteinase K. Cell wall debris, polysaccharides and remaining proteins are removed by selective precipitation with CTAB. The high molecular weight DNA is recovered from the resulting supernatant by Isopropanol precipitation.

Total genomic DNA extraction from fungi

Isolation of fungal genomic DNA was done by growing the fungi for 3-4 days. The mycelia were incubated with lysis buffer containing 250 mMTris-HCl (pH 8.0), 50 mM EDTA (pH8.0), 100 mMNaCl and 2% SDS, for 1 hr at 60°C followed by centrifugation at 12,000 rpm for 15 min. The supernatant was then extracted with equal volume of water drenched phenol and further centrifuged at 12,000 rpm for 10 min; the aqueous phase was further removed with equal volume of phenol: chloroform: isoamyl alcohol (25:24:1) and centrifuge at 12,000 rpm for 15 min; the aqueous phase was then transferred in a fresh tube and the DNA was precipitated with chilled ethanol (100%). DNA was pelleted by centrifuging at 12000 rpm for 15 min and washed in 70% ethanol by centrifugation. The pellets were air dried and suspended in TE buffer (pH 8.0) [13].

Assessing the quality of DNA by gel electrophoresis

The quality of isolated DNA was evaluated by agarose gel electrophoresis and then gel was observed under UV light in transilluminator. The DNA fluoresces under UV light due to ethidium bromide dye. The quality of DNA was judged by clarity of DNA band. The image was documented and saved in the gel documentation system.

PCR amplification using universal primers

Universal primers were designed to identify species of isolated rhizospheric bacteria; universal primers were designed using NCBI genomic sequences of *Azotobacter spp*. For amplification PCR was programmed for 94°C for 5 min, 94°C for 1 min, 40°C for 1 min, 72°C for 2 min (30 cycles). The PCR product quality was then analyzed on 1.4 % Tris acetate EDTA agarose gel pre-soaked with ethidium bromide. The profile was visualized under UV transilluminator and documented using gel documentation system BIO-RAD.

RESULTS AND DISCUSSION

Characteristics of rhizosphere soils of grape used for isolation of PGPMs

The grape rhizosphere soil samples castoff for isolation of PGPMs were analyzed for pH, EC and total organic carbon factors. Electrical conductivity was found slightly higher in Nandur Naka region; however organic carbon was significantly higher in Vani region rhizosphere soil (Table1)

Table 1: Some physio-chemical characterization of Grape Rhizosphere

Sample No.	Region	Soil type	pH	EC (dS/m)	Total Carbon
1.	Vani Region	Black	7.63 to 7.65	00.2	1.935 %
2.	Nandur Naka Region	Black	7.85 to 7.90	00.6	1.248 %

Morphological characterization

The detailed results on the morphological characterization and biochemical characterization of PGPM and fungal isolates are documented in Table 2 and Table 3 respectively. The GA and IAA production tests are positive for both of bacteria. In the IMViC all test are positive for both bacteria except voges Proskauer test i.e. negative test. The microscopic observation of fungal strains conidia are small, dark brown black round for strain one and predicted as *Aspergillus spp*; however strain two was highly branched white conidiophores under microscope are predicted as *Trichoderma* spp. (Table 4). Leelavathi et al [14] reported similar microscopic observations for *Aspergillus* and *Trichoderma* spp

DNA isolation of all two bacterial species and fungal species was carried out using miniprep method. Two colonies of each bacterial spp were used. DNA was isolation and gel electrophoresis of isolated DNA was carried out using 0.8 agarose gel to confirm the integrity of DNA. Water is used as negative control all bacterial DNA showed intact band of DNA.

Table 2: Morphological characterization of PGPM and Fungal Isolates

Sample No.	Probable Genus	Morphology	Pigmentation	Cyst Formation
1.	*Azotobacter spp*	Moderate growth, flat, entire, wrinkled at the edge of the colony	Pale light white	Present
2.	*Pseudomonas spp*	Good growth, wrinkled edges with spots	Pale yellow	Present
3.	*Aspergillus spp*	Good growth with dense and dark colony	Brown to black	Nil
4.	*Trichoderma spp*	Moderate growth with dense light spots	Whitish green	Nil

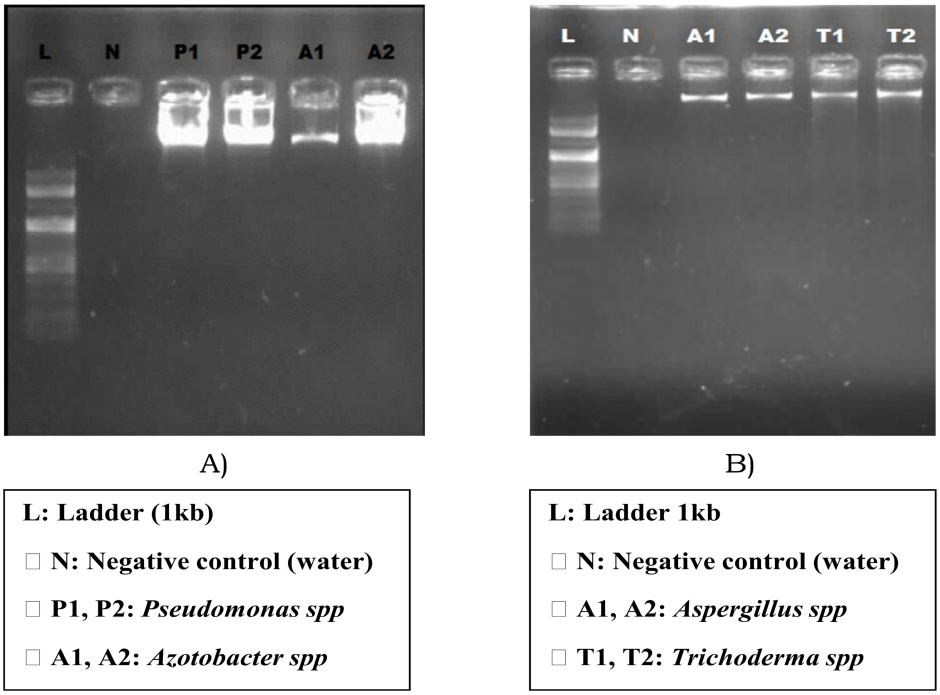
Table 3: Biochemical characterization of PGPM and Fungal Isolates

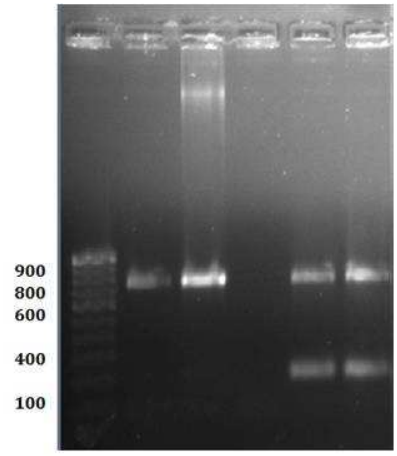
Probable genus	Cell shape	Motility test	Gram test	Catalase test	Urease test
Azotobacter spp	spiral	Non motile	Gram negative	Positive	Positive
Pseudomonas spp	rod	Non motile	Gram negative	Positive	Positive
Probable genus	**Gelatin liquefaction**	**Starch hydrolysis**	**H2S production**	**Casein hydrolysis**	**Acid production**
Azotobacter spp	Negative	Positive	Positive	Positive	Positive
Pseudomonas spp	Positive	Negative	Positive	Positive	Positive

Table 4. Microscopic Observation of Fungi

S.No	Probable Genus	Colony Morphology	Microscopic observation
1	*Aspergillus spp*	Woven thread like, black to brown spores formation and black powdery spot formation	Conidia are small, dark brown black round
2	*Trichoderma spp*	A single cottony concentric ring was found around the inoculums.	Highly branched white conidiophores formation

Figure 1: DNA isolation of Bacterial and Fungal strains in 1.4 % Agarose





L: Ladder
A1, A2, A3, A4 and A5: *Azotobacter spp*

Figure 2. 16S r-DNA characterization for *Azotobacter spp*

PCR of Azotobacter Using Universal Primers
PCR of DNA isolated from Azotobacter was carried out; universal primers for Azotobacter were used. After amplification 900bp size of 16S r-DNA of *venellandi* species was clearly found after electrophoresis using 1.2 % agarose gel.

Zone of Phosphate solubilization of (*Aspergillus spp and pseudomonas spp*) on Pikovskya's media
From grape rhizosphere soil 8 strains were screened for phosphate solubilization on Pikovskaya medium. After initial screening *Aspergillus* and *Pseudomonas* strains which show significant phosphate solubilizing zones were further grown as pure culture and then inoculated on Pikovskaya medium. *Aspergillus* and *Pseudomonas* strains show phosphate solubilizing zones of size 4-7 mm diameter when incubated for 3-4 days at 28 °C. Similar studies were reported by Karpagam and Nagalakshmi [15] showed the *Pseudomonas* and *Aspergillus* are highly efficient in solubilizing phosphate.

Figure 3. Zone of Phosphate solublization of (*Aspergillus spp and Pseudomonas spp*) on Pikovskya's media

CONCLUSION

Present study represents an attempt to isolate grape rhizosphere microflora from soil samples of two different regions of Nasik district. Plant growth promoting rhizospheric microorganisms such as *Azotobacter spp* and *Pseudomonas spp*, Phosphate solubilizing fungi *Aspergillus spp* and *Trichoderma spp*. were isolated from different grape growing They were screened for their biochemial traits like Indole (IAA) production. Biochemical characterization were done using IMViC test, Gram staining, Catalase test and Urease test and others. Both *Azotobacter spp* and *Pseudomonas spp* is IAA producer and both are Gram – ve in nature. Both *Azotobacter spp* and *Pseudomonas spp* possesses Urease production capability and both of them can help to plants for urea utilization. In morphological studies using light microscope shows that both *Azotobacter spp* and *Pseudomonas spp* are immotile in nature and morphological studies of fungi revels that *Aspergillus spp* are black and brown spore forming while *Trichoderma spp* are dark green spores and thread like structure. Molecular characterization reveals the strong presence of *Azotobacter venelandii* as reveled by 16s rDNA analysis. Further detail molecular studies of grape rhizosphere are required to understand rhizospheric plant growth promoting bacteria and their specific roles.

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