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Agriculture Importance of *Pseudomonas sp.* in Growth Promotion of Coriander Seed Spice Crop

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ABSTRACT

Agriculture Important Microorganism (AIM) as well as Plant Growth Promoting Bacteria (PGPB) are considered a promising approach to replace the conventional agricultural practices, although plant growth-promoting rhizobacteria (PGPR) have been reported to influence plant growth, yield and nutrient uptake by an array of mechanisms, the specific character by which PGPR encourage plant growth, yield and nutrient uptake were limited to the expression of one or more of the character expressed at a given surroundings of plant–microbe interaction. Since they have been shown to affect plant nutrient-acquisition processes by influencing nutrient availability in the rhizosphere and those biochemical processes determining the uptake at root level of nitrogen (N), phosphorus (P), and iron (Fe), that represent the major constraints for crop productivity worldwide. We have isolated novel bacterial strains from the rhizosphere of Coriander (Coriandrum sativum L.) seed spice crop. Rhizospheric soil sample of seed spice coriander crop was collected from Digod, Kota district of Hadoti area in Rajasthan State, India. The optimum temperature, pH and EC of collected soil sample were found to be 29ºC and 7.7, 0.08 dS/m respectively. COR-3 bacterial isolated from coriander rhizosphere soil sample and have been characterized morphological and biochemical parameters. The COR-3 isolate exhibited capacity to produce siderophores and catalase, citrase enzymes and to solubilize phosphate. COR-3 identified by molecular tools and characterized for 16S rDNA nucleotide sequencing, the result indicated that Pseudomonas putida was identified. The morphological, biochemical and molecular studies showed that Pseudomonas putida has significant potential to increase the production and to uncover its efficacy as effective agriculture important microorganism (AIM).

Key words: *Agriculture important microorganism, Pseudomonas putida, Coriander seed spice crop, 16S rDNA Sequencing, Phylogenetic study.*

INTRODUCTION

Spices are the most important and widely grown crops in the world. Spices hold prime position in the world trade market and economy.

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Enormous diversity is present in each spice crop at their geographical level as well as domestic level. The geographical and climatic peculiarities of North-West India, particularly Rajasthan and Western Gujarat region are the major gifts of nature, providing us an upper hand in the world of seed spices production. Geographical advantages of the two states coupled with the genuine efforts of the local people, help to produce the best quality spices especially seed spices which are preferred all over the world. It is widely known that the history and destiny of India, perhaps the whole world was and is influenced incredibly by the production and economic importance of seed spices.

Rajasthan has achieved considerable triumph in the export of seed spices in the last few years. Cumin, Coriander, Fennel, Fenugreek, Ajwain, Caraway, Dill, Nigella, Anise and Celery are the most important seed spices produced in India, out of which four, Cumin, Coriander, Fennel and Fenugreek are considered as major seed spices as they are playing a major role in export and foreign exchange. Coriander (*Coriandrum sativum* L.*)* is grown as seed spice crop all over the world. Coriander (*Coriandrum sativum* L.) also called cilantro, or dhania (in Hindi) is an annual herbaceous crop. Coriander is belongs to the *Apiaceae* family, formerly known as *Umbeliferaeae.*

In the era of sustainable agricultural production, the interactions in the rhizosphere play a pivotal role in transformation, enlistment, solubilization, etc. from a limited nutrient pool in the soil and consequent uptake of essential plant nutrients by the crop plants to understand full genetic potential of the crop. Soil microorganisms are very essential in the biogeochemical cycles of both inorganic and organic nutrients in the soil and in the maintenance of soil health and quality[6]. Microorganisms make up most of the biodiversity on Earth, and several of the processes which microorganisms perform are of critical importance for recycling organic materials, biogeochemical cycling of nutrients etc. hence they play an important role in maintenance of soil fertility[5]. Microbes are the key component of the soil microflora, and activities of the soil microorganisms directly or indirectly affect the nutritional status of the soil and plant health, growth and productivity. They work as bio- fertilisers, biopesticides and biocontrol agents and modulate plant immunity and health by different activities like phosphate solubilisation, nitrogen fixation, and plant growth promotion, production of plant growth- stimulating hormones and other metabolites and disease suppression[8]. Bacteria constitute one of the very valuable groups of agriculturally important microorganisms. Most of the organisms used in PGPR, biofertiliser and biopesticide formulations belong to this class. In comparison to the past, the current bacterial taxonomy is more refined and provides better resolution at species and strain levels[15]. Small subunit ribosomal RNA (16S rRNA) gene sequence-based typing and study of phylogenetic relatedness are the most popular and simple aspect of current bacterial characterization. Although 16S rRNA-based approach does not provide species level resolution in most of the cases, bacterial taxonomists usually use it as first step for typing purposes. According to the current practice, a cut-off of <95 % and <97 % 16S rRNA gene sequence similarity based on near about complete length (>1400 bp) with closely related and validly published type species is the criteria for the creation of new genus and species, respectively[20].

MATERIAL AND METHODS

Sample collection and isolation

A set of 4 soil samples used in the present study were collected from the rhizospheric soil of *Coriandrum sativum* plants from Digod, Kota district of Hadoti region in Rajasthan state in the month of December 2015 and were properly stored at 4° C in the refrigerator at the laboratory of Pacific academy of higher education and research university, Udaipur and were used for morphological, biochemical and molecular analysis. Collected rhizospheric soil samples were used for isolating the rhizospheric bacterial isolates using serial

dilution method. The serially diluted soil suspension was spread on various media which include nutrient agar medium, King's B agar medium[10], Pikovaskaya agar medium[13], Simmon's citrate agar medium[16], Jensen's agar medium[7] and Cristensen's urea agar medium. The purified bacterial isolates were preserved in 20% glycerol at 20° C.

Morphological and Biochemical characterization for agriculturally important traits of isolated bacterial strain

Morphological characters of the selected strain were analyzed which included, color, form, elevation, margin, consistency, sedimentation in nutrient broth, gram's staining, endospore staining and shape. The Gram's staining and endospore staining was performed as per standard procedures[17].

For detection of catalase enzyme a small amount of a bacterial culture (18 to 24 hours old) was placed by flame sterilized inoculating loop on a clean grease free glass slide then added one to two drops of 3% H_2O_2. Observations were recorded. For detection and quantitative analysis of IAA, isolated bacterial culture was inoculated in presterilized tryptone broth, which contains amino acid tryptophan. Culture tubes were incubated overnight at 37°C. After incubation few drops of Kovac's reagent were added to the broth containing bacteria. Culture tubes were observed for the appearance of cherry red color. This indicated positive test for production of indole.

Citrate Utilization test detects the ability of an organism to utilize citrate as the sole source of carbon and energy. Bacterial colonies were picked up with a straight wire and inoculated into slant of Simmon's citrate agar medium containing sodium citrate and a pH indicator bromothymol blue. Inoculated culture tubes were incubated overnight at 37°C. Utilization of citrate involves the enzyme citrase, which breaks down citrate to oxaloacetate & acetate. Oxaloacetate was further broken down to pyruvate and CO_2. Production of Na_2CO_3 as well as NH_3 from utilization of sodium citrate and ammonium salt respectively results in alkaline pH. This results in change of medium's color from green to blue[5].

HCN production- The bacterial cultures were streaked on King's B agar medium amended with 4.4 g glycine. Circular whatman (No. 1) filter paper soaked in 2% sodium carbonate in 0.5% picric acid solution was placed in the top of the plate. Plates were sealed air tight with parafilm and incubated at 28 º C for 4 days. The HCN production was indicated by the development of orange to red color from yellow.

Phosphate solubilization test-The plates were prepared with presterilized Pikovaskaya's medium. All the isolates were tested for their ability to solubilize phosphate using Pikovaskaya's medium. The culture was inoculated on the Pikovskaya medium plates and incubated at temperature 28°C for 7 days. Formation of clear zone around bacterial growth was taken as a positive test.

Siderophore Production- chrome azurol-S (CAS) assay described by Schwyn and Neilands[14] was used for the qualitative estimation of the siderophores production. CAS agar plates were prepared and poured with the sterilized media. The isolated bacterial colonies were inoculated on the petri plates and incubated at 30 º C for 3-5 days. Development of a yellow-orange halo around the colony was considered as a positive result.

DNA isolation, PCR amplification and Nucleotide sequencing

Pure culture of the isolated bacteria COR-3 was raised in 10 ml of luria bertani broth for 24 hours to obtain O. D. at 600nm. The culture broth 1.5 ml was pelleted in a micro centrifuge tube for 2 min at 12000 rpm. The isolated bacterial culture and pellet form was sent to the B. Lal Institute of Biotechnology, Jaipur for DNA isolation, PCR amplification and 16S rDNA sequencing. Genomic DNA was isolated from the culture COR-3. Quality was evaluated on 0.8% agarose Gel, a single band of high-molecular weight DNA has been observed.

Amplification of 16S rDNA was carried out by polymerase chain reaction using a thermal cycler. The following programmed was used for the amplification of 16S rDNA, The reaction was performed at 94º C for 3

min, 55 º C for 1 min and 72 º C for 1 min followed by a final extention of 10 min at 72 º C. Removed unincorporated PCR primers and dNTPs from PCR products by using Montage PCR Clean up kit (Millipore).The PCR product was sequenced using the 27F/1492R primers. Sequencing reactions were performed using a ABI PRISM® BigDyeTM Terminator Cycle Sequencing Kits with AmpliTaq® DNA polymerase (FS enzyme) (Applied Biosystems).

Single-pass sequencing was performed on each template using below 16s rRNA universal primers. The fluorescent-labeled fragments were purified from the unincorporated terminators with an ethanol precipitation protocol. The samples were resuspended in distilled water and subjected to electrophoresis in an ABI 3730xl sequencer (Applied Biosystems). The 16s r RNA sequence was blast using NCBI blast similarity search tool. The phylogeny analysis of query sequence with the closely related sequence of blast results was performed followed by multiple sequence alignment.

RESULT AND DISCUSSION

Selection of an effective PGPR was based on a wide range of attributes, making them suitable for diverse environments and soil types. In our study about 7 bacterial isolates were picked on the basis of their morphology and colony characteristics. The morphology of the isolates varied largely. Bacterial colonies had different forms, shapes and elevations. The color of isolated colonies ranged from white, off white, yellow, pink, orange and creamish. The colony surfaces of bacterial isolates were varied from smooth shiny to rough appearance. *In vitro screening* of plant growth-promoting (PGP) traits was carried out using eight *Pseudomonas* spp., PPR1 to PPR8, isolated from the rhizosphere of *Phaseolus vulgaris* growing on the Uttarakhand Himalayan range in India. All the isolates were fast growers, positive for catalase, oxidase and urease activities, and utilized lactose and some amino acids. All the isolates were indole acetic acid (IAA) positive, however PPR8 solubilized potassium and zinc along with various other types of inorganic (tricalcium, dicalcium and zinc phosphate) and organic (calcium phytate) phosphates, as well as producing siderophore and ACC deaminase[12].

Morphological analysis of the strain- The isolated strain was gram negative, short rod shaped with no endospore formation. The bacteria isolate COR-3 had entire margin and flat elevation. The colony was white in colour with smooth and shiny appearance. (Table 1)

PGPB have been identified by molecular tools and characterized for their capacity to produce siderophores and indole-3-acetic acid (IAA), and to solubilize phosphate (Table 2). Selected bacterial isolates, showing contemporarily high levels of the three activities investigated, were finally tested for their capacity to induce Fe reduction in cucumber roots two isolates, from barley and tomato plants under Fe deficiency, significantly increased the root Fe-chelate reductase activity; interestingly, another isolate enhanced the reduction of Fe-chelate reductase activity in cucumber plant roots, although grown under Fe sufficiency[11]. In the present study siderophore production was shown by forming an orange hallo zone around the culture colony growth on CAS medium (Table 2). Arif *et al.* [1] demonstrated that siderophore production by rhizobia showed better plant growth with respect to increase in root weight, shoot weight and chlorophyll.

Identification- Single-pass sequencing was performed on each template using below 16s rRNA universal primers. The fluorescent-labeled fragments were purified from the unincorporated terminators with an ethanol precipitation protocol. The COR -3 sample was resuspended in distilled water and subjected to electrophoresis in an ABI 3730xl sequencer (Applied Biosystems).

On the basis of biochemical and nucleotide homology and phylogenetic analysis the isolate COR-3 was showed maximum similarity with *Pseudomonas putida strain* TDRl3. The 16S rDNA sequence was used to carry out BLAST alignment search tool of NCBI genbank database. Based on maximum

identity score, sequences were selected and aligned using multiple alignment software program Clustal W. Distance matrix was generated and the phylogenetic tree was constructed using MEGA5 (Fig 1).

The evolutionary history was inferred using the Neighbor-Joining method[14]. The bootstrap consensus tree inferred from 1000 replicates is taken to represent the evolutionary history of the taxa analyzed[4]. Branches corresponding to partitions reproduced in less than 50% bootstrap replicates are collapsed. The evolutionary distances were computed using the Jukes-Cantor method[9] and are in the units of the number of base substitutions per site. The rate variation among sites was modeled with a gamma distribution (shape parameter = 1). The analysis involved 16 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated. There were a total of 1404 positions in the final dataset. Evolutionary analyses were conducted in MEGA5[19].

Accession number- The sequence was submitted to the National Centre for Biotechnology Information (NCBI) and is available under the Genbank accession no. KY810614.

Based on the agriculture important traits of all 7 bacterial isolates, COR-3 was found to be the most potent plant growth-promoting rhizobacteria (PGPR). Further, COR-3 was identified as *Pseudomonas* putida COR-3, based on 16S rRNA gene sequencing analysis (Fig 1). Moreover, the plant growth promoting activities of COR-3 confirmed it to be a potent Plant growth promoting rhizobacteria. This study reveals the potential of *Pseudomonas* putida COR-3 to be used as a good bioinoculant for growth promotion of coriander seed spice and for the protection of important seed spice crops from various deleterious phytopathogens.

Table 1: (Morphological characterization of isolated agriculture important bacteria COR 3)

Isolate strain	Gram's staining	Endospore Staining	Shape	Colour	Margin	Elevation	Consistency	Form
Pseudomonas putida COR-3	-	-	Short rod	White	entire	Flat	Smooth and shiny	circular

Table 2: (Biochemical characterization of isolated agriculture important bacterial isolate COR 3)

Isolate strain	Catalase	Amylase	Oxidase	IAA	Phosphate solubilization	Citrate utilization	HCN production	Siderophore production
Pseudomonas putida COR-3	+	+	+	+	+	+	-	+

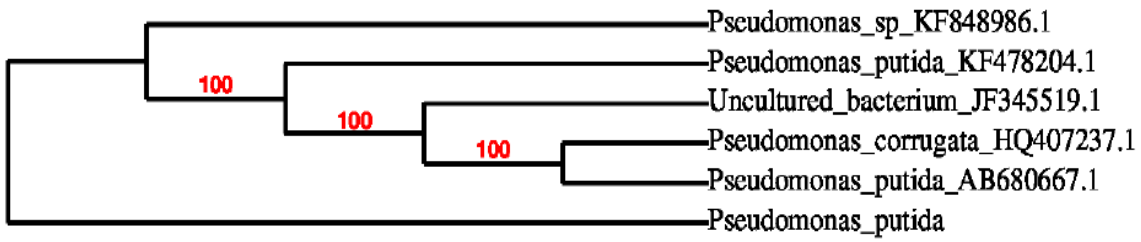
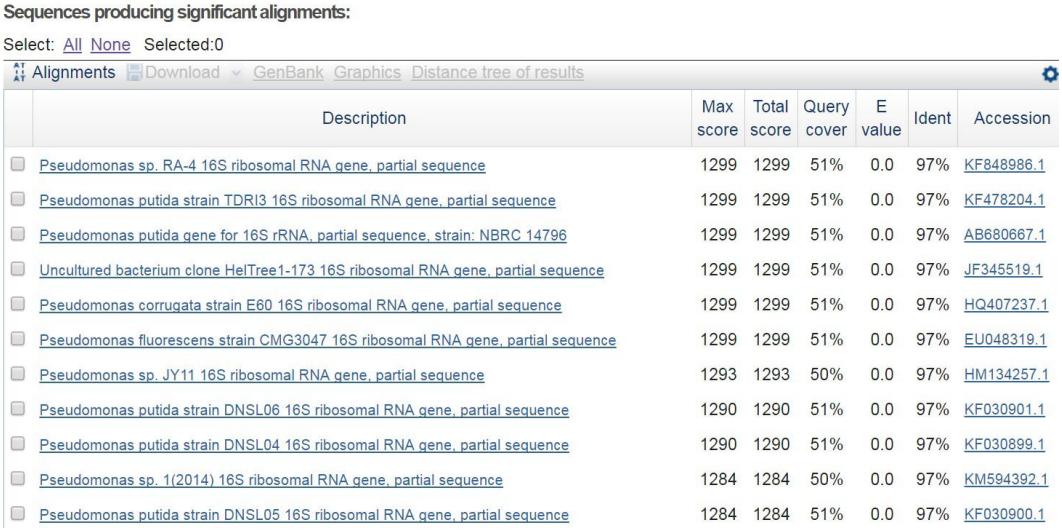


Fig. 1: Phylogenetic relationship based on 16S rDNA sequences. The agriculture important bacterial isolate COR-3 with other closely related bacterial isolated strains resulted from Blast tool.



Sequences producing significant alignments:

Select: All None Selected:0

Alignments Download GenBank Graphics Distance tree of results

Description	Max score	Total score	Query cover	E value	Ident	Accession
Pseudomonas sp. RA-4 16S ribosomal RNA gene, partial sequence	1299	1299	51%	0.0	97%	KF848986.1
Pseudomonas putida strain TDRI3 16S ribosomal RNA gene, partial sequence	1299	1299	51%	0.0	97%	KF478204.1
Pseudomonas putida gene for 16S rRNA, partial sequence, strain: NBRC 14796	1299	1299	51%	0.0	97%	AB680667.1
Uncultured bacterium clone HelTree1-173 16S ribosomal RNA gene, partial sequence	1299	1299	51%	0.0	97%	JF345519.1
Pseudomonas corrugata strain E60 16S ribosomal RNA gene, partial sequence	1299	1299	51%	0.0	97%	HQ407237.1
Pseudomonas fluorescens strain CMG3047 16S ribosomal RNA gene, partial sequence	1299	1299	51%	0.0	97%	EU048319.1
Pseudomonas sp. JY11 16S ribosomal RNA gene, partial sequence	1293	1293	50%	0.0	97%	HM134257.1
Pseudomonas putida strain DNSL06 16S ribosomal RNA gene, partial sequence	1290	1290	51%	0.0	97%	KF030901.1
Pseudomonas putida strain DNSL04 16S ribosomal RNA gene, partial sequence	1290	1290	51%	0.0	97%	KF030899.1
Pseudomonas sp. 1(2014) 16S ribosomal RNA gene, partial sequence	1284	1284	50%	0.0	97%	KM594392.1
Pseudomonas putida strain DNSL05 16S ribosomal RNA gene, partial sequence	1284	1284	51%	0.0	97%	KF030900.1

Fig. 2: Alignment view of *Pseudomonas putida* COR-3 using combination of NCBI GenBank

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