

## Plant Growth Promoting Rhizobacteria of *Curcuma amada* (Mango ginger)

Ajay Kumar, Hariom Verma, Akhilesh Yadav, Waquar Akhtar Ansari,  
Prem Pratap Singh, Sandeep Kumar Singh, P.K. Singh and K.D. Pandey\*

Centre of Advance study in Botany, Banaras Hindu University, Varanasi - 221 005, India.

<http://dx.doi.org/10.22207/JPAM.11.1.67>

(Received: 15 August 2016; accepted: 30 September 2016)

In this study, 31 bacterial strains were isolated from the rhizospheric soil of *Curcuma amada* (mango ginger) and their plant growth promotion potential, salinity tolerance, antibiotic sensitivity, antimicrobial properties were evaluated. Eight bacterial strains namely *Azotobacter chroococcum* KCA1, *Pseudomonas fluorescens* KCA2, *Bacillus subtilis* KCA3, *Bacillus* sp. KCA4, *Agrobacterium tumefaciens* KCA5, *Bacillus cereus* KCL7, *Pseudomonas putida* KCA8 and *Paenibacillus* sp. KCA9 have been identified on the basis of biochemicals and 16S r RNA gene sequence analysis. All the strains solubilized tri-calcium phosphate and produced IAA, ammonia but only 50% of the strains produced siderophores during PGP traits analysis. Strains KCA8 tolerated maximum NaCl (7%) relative to strain KCA5 (1-2%). The strains were sensitive to the antibiotic chloromphenicol followed by erythromycin and most of these effectively inhibited growth of *Escherichia coli*, *Fusarium solani* and *Alternaria alternata* during antimicrobial properties.

**Keywords:** *Curcuma amada*, PGPR, PGP traits, Antimicrobial properties, Salinity tolerance.

Plants growth promoting rhizobacteria (PGPR) are the heterogeneous group of bacteria that effectively colonize roots and exerting plants growth promotion. These PGPR directly or indirectly involved in morphological growth, enhancement in the secondary metabolite production of the plants (Kumar *et al.*, 2016b). PGPR enhance plant growth directly through the production of siderophore, N<sub>2</sub> fixation, phosphate solubilization, synthesis of plant growth hormones like auxin, cytokinin, gibberellins (Lucy *et al.*, 2004) and also by increasing the uptake of water and minerals (Perez-Montano *et al.*, 2014). Recently,

PGPR along with certain level of fertilizers have been broadly used as plants or soil inoculants in sustainable agriculture because of their less adverse impact on the soil, plants and environment (Lucy *et al.*, 2004; Perez-Montano *et al.*, 2014). Numerous studies has been carried out from last two decades in the field of isolation, characterization of PGPR and their uses as soil and plants inoculants for the growth, yields and disease management (Goswami *et al.*, 2013; Perez-Montano *et al.*, 2014; Kumar *et al.*, 2014, 2015, 2016b).

*Curcuma amada* (mango ginger) member of family Zingiberaceae is a spice and well known traditional medicine in the Ayurveda since ancient time in the Indian sub-continent (Sasikumar, 2005). Plant extensively used in foods as prickles, salad etc (Shankaracharya, 1982) and in pharmacology broadly used as antioxidant, anti-inflammatory, treatment of flatulence, jaundice, menstrual

\* To whom all correspondence should be addressed.  
Tel.: 91-05426701104; Fax No-91-542-2368174;  
E-mail [kdpandey2005@gmail.com](mailto:kdpandey2005@gmail.com)

difficulties, hemorrhage and colic (Mujumdar *et al.*, 2000; Prakash *et al.*, 2007; Policegoudra *et al.*, 2007). The rhizome of *Curcuma amada* have also cancer preventive or therapeutic capabilities. It has been shown to suppress multiple signaling pathways and inhibit cell proliferation, invasion, metastasis and angiogenesis (Policegoudra, 2008). Curcuminoids are the important constituents of mangoginger. Amongst curcuminoids, curcumin is the most important constituent (Gupta *et al.*, 1999). The researches carried out in the past half century in this field clearly indicated the importance of curcumin in pharmacology.

The objective of this study is to isolate bacterial strains from the rhizospheric soil of *Curcuma amada* and to access their plant growth promotion potential, salinity tolerance as well as antimicrobial properties.

## MATERIALS AND METHODS

### Soil sampling and bacterial isolation

Bacterial strain were isolated from the rhizospheric soil of young and healthy mangoginger (*Curcuma amada*) grown in the Botanical garden of Banaras Hindu University, India (20° 18' N and 80° 36' E, elevation 80.71m) using standard microbiological techniques (soil serial dilutions or spread-plate methods). Rhizospheric soil (1g) was dissolved in 10 ml of sterile distilled water, making 10<sup>-1</sup> dilution. This dilution was further diluted to 10<sup>-7</sup>. 1 ml of each dilution 10<sup>-6</sup> and 10<sup>-7</sup> was placed on nutrient agar, in triplicate (Kumar *et al.*, 2016b) for total bacterial counts using King's B medium for *Pseudomonas* sp. and N<sub>2</sub> free agar medium for *Azotobacter* sp. The plates were incubated (48h) at 30°C and colonies showing morphological difference, separately isolated for further analysis (Kumar *et al.*, 2016b). Further characterizations of bacterial isolates were performed on the basis of morphology and biochemical screening according to Bergey's manual of systematic bacteriology (Holt *et al.*, 1994).

### Identification of bacterial isolates by 16S rRNA amplification

#### 16S r DNA sequence amplification and sequencing

Genomic DNA was isolated using GeneiPure™ bacterial DNA purification kit

(GeNei™, Bangaluru, India) according to the manufacture's protocol. Universal eubacterial primers F-D1-5'-ccgaattcgtcgacaacagattgatcctggctcag-3' and R-D1-5'-cccgggatccaagcctaaggaggatccagcc-3' (Kumar *et al.*, 2015, 2016a, b), were used to amplify the 1500 bp region of 16S rRNA gene using a thermal cycler (Bio Rad, USA). Amplified products were resolved by electrophoresis in agarose (1%), and visualized in the gel documentation system (Alfa Imager, Alfa InfoTech Corporation, USA). The amplicons were purified using GeneiPure™ quick PCR purification kit (GeNei™, Bangaluru, India) and quantified at 260 nm taking calf thymus DNA as control. The purified partial 16S rDNA amplicon was sequenced in Applied Biosystems 3130 Genetic Analyzer (Applied Biosystems®, CA, and USA).

#### Analysis of 16S r RNA gene sequences

The 16S r RNA gene partial sequences of the isolated strains were sequenced and compared with the RNA databases. The resulting nucleotide sequence were assigned for bacterial taxonomic affiliation based on the closest match to the sequences available at National Center for Biotechnology Information (NCBI) BLAST server ([www.ncbi.nlm.nih.gov/BLAST](http://www.ncbi.nlm.nih.gov/BLAST)) using Nucleotide Basic Local Alignment Search Tool (BLAST) program (Kumar *et al.*, 2015, 2016a, b)

#### Plant growth promoting (PGP) traits of bacterial isolates

The bacterial isolates were screened for their plant growth promoting potential including phosphate solubilization (Laslo *et al.*, 2012), Indole acetic acid production (IAA) (Brick *et al.*, 1991), siderophore production (Schwyn and Neilands, 1987) and NH<sub>3</sub> production (Cappuccino and Sherman, 1992) as per the standard protocols.

#### Antibacterial activity

All the rhizospheric strains were screened for antibacterial activity against *Escherichia coli*, *Pseudomonas aeruginosa* and *Klebsiella pneumoniae*. The bacterial strains were grown on nutrient agar plates and inoculated with single streak at the center of the petri-plate and incubated at 30°C for 2-4 days. 24 h old growth cultures of the isolated rhizospheric strains were streaked on the test organisms to observe their growth or inhibition after 24-48 h of incubation (30°C). The diameters of the inhibition zone were measured in mm (Kumar *et al.*, 2015, 2016 a, b).

### Antifungal activity

All the eight rhizospheric strains were tested against four moulds *Fusarium solani*, *Alternaria alternata*, *Byssoschlamys fulva* and *Aspergillus fumigates* to check their fungistatic activity. A drop of the 24 h old cultures of selected bacterial strains grown in nutrient broth were placed separately in a row on the fungal test cultures prepared in Potato Dextrose Agar (PDA) medium. The plates were incubated at room temperature (3-5 days) and inhibition zone exhibit the fungistatic activity of the bacterial strains (Kumar et al., 2015, 2016a, b).

## RESULTS AND DISCUSSION

In the rhizospheric region of mango-ginger 31 bacterial isolates were isolated, all the strains were rod shaped and most of them were Gram negative (62.5%). All the rhizospheric isolates were positive for catalase and oxidase test. The isolated bacterial strains belongs to different genera namely *Azotobacter* (6), *Pseudomonas* (8), *Agrobacterium* (3), and *Bacillus* (14) of phyla  $\alpha$ -Proteobacteria,  $\beta$ -Proteobacteria and Firmicutes on the basis of morphology and biochemical characteristics (Table 1). Further confirmations of the species levels of the isolates were carried out through 16S r RNA gene sequence analysis. Rhizobacterial isolates were identified as 8 different bacterial strains and their sequence were deposited in the NCBI and got accession no. namely *Azotobacter chroococcum* KCA1 [accession no. KM043465], *Pseudomonas fluorescens* KCA2 [accession no. KMO43460], *Bacillus subtilis* KCA3 [accession no. KM043461] *Bacillus* sp. KCA4 [accession no. KM043462], *Agrobacterium tumifaciens* KCA5 [accession no. KMO43463], *Bacillus cereus* KCA7 [accession no. KMO43464], *Pseudomonas putida* KCA8 [accession no. KM043466] and *Paenibacillus* sp. KCA9 [accession no. KMO43467].

Plant interacts with diverse communities of microorganism, rhizosphere is the most prominent zone for growth and interactions among the microbes mainly due to the secretion of root exudates. In the previous study it is reported that in the rhizospheric zone the predominant bacterial species belong to phyla  $\alpha$ -proteobacteria,  $\beta$ -proteobacteria,  $\gamma$ -proteobacteria and firmicutes,

rhizodeposition predominantly colonized the Gram negative microbial community (Ambardar and Vakhlu, 2013; Kumar et al., 2016b). In this study, mango-ginger rhizome was also dominated by Gram negative (62.5%) and proteobacteria phyla.

### Plant growth promoting (PGP) traits

Plant growth promoting traits mainly includes synthesis of phytohormones, phosphate solubilization, production of siderophores, these attributes helps in plant growth. All the bacterial isolates produced IAA, ammonia and solubilized tri-calcium phosphate on Pikoskaya nutrient agar petriplates, where as only 50% strains produced siderophores (Table 1). Phytohormones produced by plant-associated bacteria are mainly indole-3-acetic acid (IAA), cytokinin and gibberellins which frequently stimulate plant growth and protect against biotic and abiotic stresses (Taghavi et al., 2009). All the eight rhizobacterial strains synthesized IAA in presence of tryptophan. Siderophore is an iron-chelating compounds secreted by certain PGPR strains. The presence of iron-chelating compounds makes the bacteria better competitors for iron that prevents growth of pathogenic microorganisms. Plants which unable to uptake sufficient amount of iron benefitted from siderophore producing bacteria that chelate  $Fe^{+3}$  and make it available to plants for growth. In a previous study, phosphate solubilization, IAA and siderophore production are already reported in *A. chroococcum*, *Pseudomonas* sp. (Maheshwari et al., 2012; Laslo et al., 2012; Kumar et al., 2016b), *Agrobacterium tumifaciens*, *Bacillus* sp. and *Pseudomonas* sp. by (Zhao et al., 2014; Kumar et al., 2016b).

Most of the rhizobacterial strains shown tolerance to salinity upto 4% of NaCl except *A. tumifaciens* KCA5 (1-2% NaCl). Strains *Paenibacillus* sp. KCA9 shown tolerance upto 5% of NaCl, Strains *P. fluorescens* KCA2, *B. subtilis* KCA3, *Bacillus cereus* KCA7 tolerated salinity upto 6% of NaCl, whereas *P. putida* KCA8 shows maximum salinity tolerance (7% of NaCl) (Table 1).

Salinity is one of the most severe abiotic stresses that limits crop growth and productivity. Under salt stress, PGPR have positive effects on plants or parameters like germination rate, tolerance to draught and development of shoots and roots.

In the present study, *P. putida* exhibit maximum salinity tolerance contrary to the minimum

**Table 1.** Biochemical characterization of selected bacterial isolates from *C. amada* rhizosphere. (+ve-Positive, -ve-Negative)

Bacterial strain	Gram staining	Shape	Catalase	Oxidase	Nitrate reduction	H <sub>2</sub> S Production	Starch hydrolysis	Salinity tolerance	Phosphate solubilization	IAA Production	Ammonia production	Siderophore production
KCA1	-	Rod	+	+	+	-	+	2%	+	+	+	-
KCA2	-	Rod	+	+	+	-	+	6%	+	+	+	+
KCA3	+	Rod	+	+	-	+	+	6%	+	+	+	-
KCA4	+	Rod	+	+	+	-	+	5%	+	+	+	+
KCA5	-	Rod	+	+	+	+	+	1%	+	+	+	-
KCA7	+	Rod	+	+	+	-	+	5%	+	+	+	+
KCA8	-	Rod	+	+	-	-	+	7%	+	+	+	+
KCA9	-	Rod	+	+	-	-	+	5%	+	+	+	-

**Table 2.** Antibiotic sensitivity, antibacterial activity and antifungal activity of rhizospheric strains of *C. amada*.

Bacterial strain	Chloramphenicol	Erythro-mycin	Antibiotic sensitivity (antibiotics inhibition zone in mm)		Antibacterial activity (inhibition zone in mm)	Antifungal activity (fungal growth inhibition)	Siderophore production
			Rifam-picin	Polymixin-B			
KCA1	28	25	15	11	-	+	-
KCA2	19	16	-	-	-	+	+
KCA3	22	19	9	-	+9	+	+
KCA4	26	21	10	8	+7	+	+
KCA5	24	22	12	10	-	+	-
KCA7	26	20	10	9	+9	+	+
KCA8	22	16	-	-	+12	+	+
KCA9	25	18	-	-	-	+	-

(1-2% NaCl) in *A. tumefaciens*. Already more or less similar trends of salinity tolerance was reported by Kumar *et al.* (2015, 2016b). In a study Rashid *et al.* (2012) reported differential salinity tolerance pattern of the strains *P. fluorescens* (4% NaCl), *Bacillus* sp. (3.5% NaCl) and *A. tumefaciens* (0.5-1% NaCl). *Bacillus* sp. isolated from marshy areas had tolerance upto 10% of NaCl (Gayathri *et al.*, 2010). Singh *et al.* (2013) also reported tolerance 4-10 % of NaCl in case of *Momordica charantia* bacterial isolates.

#### Antibiotic sensitivity

The sensitivity pattern of antibiotic disc towards bacterial isolates were determined by disc diffusion method. All the four antibiotics showed differential pattern of inhibition as results showed in Table 2. Strains were mostly sensitive to chloramphenicol followed by erythromycin while some strains were resistant to rifampicin and polymixin-B. Strains *P. fluorescens* KCA2, *B. subtilis* KCA3 and *P. putida* KCA9 showed high resistance against two antibiotic (rifampicin and polymixin-B) whereas strains *A. tumefaciens* KCA5, and *A. chroococcum* KCA1 were highly susceptible to all the antibiotics tested. The antibiotic disc acted differentially on growth of the same bacterial strains of different isolation source (Arunachalam and Gayathri, 2010; Kumar *et al.*, 2015; 2016b).

#### Antibacterial and antifungal activity

All the rhizobacterial strains were assessed for antibacterial property against *E. coli*, *P. aeruginosa* and *K. pneumoniae*. The isolates which inhibited the growth of any test organism(s) was considered having antibacterial activity and the diameter of inhibition zone was measured in mm (Table 2). All the strains showed antibacterial activity against *E. coli* except *A. chroococcum* KCA1 and *A. tumefaciens* KCA5. Strains *P. fluorescens* KCA2, *B. subtilis* KCA3 and *P. putida* KCA8 were antibacterial against *K. pneumoniae*, whereas none of the strains inhibited growth of *P. aeruginosa*.

During antifungal activity, all the rhizobacterial strains exhibited antifungal property with the exception of *A. chroococcum* KCA1, *A. tumefaciens* KCA5 and *Paenibacillus* sp. KCA9 that did not show fungistatic activity against *B. fulva* and *A. fumigates* (Table 2). In the previous study, it is reported that some of the bacterial strains are a rich source of bioactive natural

compounds like Ecomycins, Pseudomycins and Kakadamycins, which contains antibacterial and antifungal properties (Christina *et al.*, 2013) and play significant role in antibacterial and antifungal properties. In the present investigation, *P. fluorescens* (KCA2), *B. subtilis* (KCA3) *B. cereus* (KCA7) and *P. putida* (KCA8) formed inhibition zones against most of the fungal strains. In antibacterial activity tests, all the strains inhibited growth of *E. coli* except strains *A. chroococcum* KCA1, *A. tumefaciens* KCA5 and *Paenibacillus* sp. KCA9. These three strains also showed poor activity during antibiotic tests.

The fungal strains used during the antifungal activity test are the potent pathogens and causes severe infection to plant species as well as on the living organisms. *Fusarium solani* is a filamentous fungus commonly isolated from the soil and plant debris. *A. alternate* causes several diseases on the plants like leaf spot. It is an opportunistic pathogen on numerous hosts causing 'leaf spots', 'rots' and 'blights' on many plant parts. *B. fulva* is responsible for fruit rot in certain plants. *A. fumigatus*, a saprotroph, is widespread in nature, and typically found in soils and decaying organic matter (Kumar *et al.*, 2016b). The antifungal activity of the strains isolated is attributed to the secretion of lytic enzymes, chitinase and certain antibiotics. Our finding show that all the bacterial isolates possess antifungal characteristics against above fungal strains except the strain *A. chroococcum* KCA1 and *A. tumefaciens* KCA5 that did not form inhibition zone against *A. Niger* or *B. fulva*.

Environment friendly sustainable agriculture is preferred to obtain the desirable yield. In this respect, PGPR are applied in a wide variety of agro and allied industries as inoculants and considered promising for managing soil fertility and plant growth (Aarons *et al.*, 2000). This study shown that strains *P. fluorescens* KCA2, *P. putida* KCA8 and *Bacillus* strains may be proven as better choice in the sustainable agriculture as plant or soil inoculants.

#### ACKNOWLEDGEMENT

Author Ajay Kumar, thankful to University Grant Commission, New Delhi for financial assistance in the form of JRF and SRF

and also to Head, Department of Botany for providing lab facilities.

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