

Isolation and Characterization of Antagonist Bacteria from Chickpea Rhizosphere against *Fusarium oxysporum* f. sp. *ciceris*

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Fourteen potential antagonist bacterial isolates were obtained from soil samples collected from the rhizospheric soil of healthy chickpea. Colony and morphological characterization revealed that all isolates were diversified and distinct from each other. The antagonism characters against *Fusarium* wilt pathogen of these isolates were very prominent and ranged from 21.7 to 75.0%, seven out of fourteen isolates were showing more than 50.0% inhibition of *Fusarium oxysporum* under *In vitro* assay. These seven highly potential bacterial isolates were identified by 16s rRNA sequencing out of which three highly effective antagonistic isolates were identified as *Burkholderia* sp. (K10), *Bacillus subtilis* (K18) and *Bacillus aryabhatai* (K21) showing 71.2, 75.0 and 70.2% antagonism, respectively. The plant growth promoting characters of all the isolates viz., nitrogen fixation, phosphate solubilization, zinc solubilization, siderophore production and IAA production were studied. Highly effective antagonist isolates K18 and K21 were also efficient for N₂ fixation and Zn solubilization. These two promising antagonist isolates with PGPR activities could be further evaluated in field study for their commercial exploitation.

Keywords: Antagonist, biocontrol, *Fusarium oxysporum* f. sp. *ciceris*.

Rhizosphere region harbours bewildering diversity of different group of microorganisms. It is a potential place for complex plant-microbe and microbe-microbe interactions¹. Thus, rhizosphere region is an extremely complex habitat for microbes known as Plant Growth Promoting Rhizobacteria (PGPR), plant beneficial bacteria. Many PGPR helps plant to impart resistance to biotic stresses by antagonism mechanism i.e. interference with plant pathogen growth, survival, infection or plant attack². Antagonistic bacteria can inhibit plant pathogens by different mechanisms like inhibition of the pathogen by antibiotics and toxins,

competition for space and nutrients, parasitism by extracellular cell-wall-degrading enzymes, mycophagy, etc^{1,3}.

Chickpea (*Cicer arietinum* L.) is pivotal source of protein for vegetarian diet, however the productivity of chickpea is far below its potential⁴. One of the major reasons for the low productivity of cultivated chickpea is its narrow genetic bases and its sexual incompatibility with other wild species of *Cicer* in natural inter specific cross. Furthermore, various biotic (*Fusarium* wilt, *Ascochyta* blight, nematodes and pests) and abiotic (heat, salinity, drought and cold) stresses severely reduce the yield⁵. Among the biotic stresses, the *Fusarium* wilt and *Ascochyta* blight are the most important fungal diseases causing serious yield losses. *Fusarium* wilt caused by

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Fusarium oxysporum f. sp. *ciceris* (Padwick) Matuo & K. Sato (FOC) is one of the most important and destructive vascular disease of chickpea. *Fusarium* wilt is primarily managed by resistance breeding programs but pathogenic variability and mutability leading to breakdown of naturally selected resistance, are the main hurdles for plant breeders⁶. The fungicides are used for wilt management however, the degradation of fungicides and organic compounds are very difficult and it's accumulation in food chains had higher toxic level in animals and also lead to environment pollution⁷. Therefore, Integrated Disease Management (IDM) strategy is need of time which includes minimum use of chemicals for checking the pathogen population, encouragement of beneficial biological agents to reduce the pathogen inoculums⁸, further the crop rotation, pathogen-free seed, removal of plant debris and fungicide seed treatment are several disease management strategies have been employed for the control of wilt in chickpea. Biological control of *Fusarium* wilt is a potential component of IDM where the principle of bacteria or fungal antagonist has been exploited for the control of disease. The application of microorganisms to control diseases, which is a form of biological control, is an environment friendly approach. Realizing the need of novel microbial biocontrol agent against the *Fusarium* wilt of chickpea, the present study is undertaken with an objective to isolate efficient biocontrol agent with PGPR activity from chickpea rhizosphere.

MATERIALS AND METHODS

Collection of samples

Different soil samples were collected from the rhizosphere region of healthy chickpea plant from pulse research station, Navsari Agricultural University, Navsari 396 450, Gujarat, India.

Isolation of PGPR

Collected soil samples were serially diluted, plated on N-agar medium and incubated for 24 hours. After incubation, well isolated and distinct colonies were streaked on N-agar plates by four sector method⁹. Antagonist effect of all isolates was studied against *F. oxysporum* following the method described by Jinantana and Sariah¹⁹⁹⁷¹⁰. Based on initial screening for

antagonism against *Fusarium oxysporum* f. sp. *ciceris*, total fourteen distinct isolates showing considerable antagonism were preserved on agar slant at 4°C and glycerol stocks were prepared for long term preservation of isolates.

Characterization of isolates

All the isolates were characterized in terms of colony and morphological characters to judge diversity and distinctness among isolates.

In vitro screening of isolates

Isolates were screened for their antagonist as well as plant growth promoting activities such as nitrogen fixation, phosphate and zinc solubilization, siderophore production and IAA production.

Antagonistic activity

Antagonistic activities of the isolates were evaluated by dual culture method against *Fusarium oxysporum* f. sp. *ciceris*. Fungal and bacterial isolates were inoculated on Potato Dextrose Agar (PDA) medium in test petri dish and inoculation of only fungal pathogen on PDA served as a control. Plates were incubated at 30°C temperatures till full coverage of fungi in control. Efficiency of isolates was judged on per cent growth inhibition method given by Vincent, 1947¹¹.

$$PGI = \frac{C-T}{C} \times 100$$

Where,

PGI= Percent Growth Inhibition

T= Colony diameter of pathogenic fungi in test plate (mm)

C= Colony diameter of pathogenic fungi in Control plate (mm)

Nitrogen fixation

Nitrogen fixation ability of isolates was judged by inoculating pure culture on nitrogen free Ashby's Mannitol Agar medium. Presence of growth after 48 hrs of incubation at 28°C temperature was taken as evidence of positive nitrogen fixation reaction.

Phosphate solubilization

Rate of phosphate solubilization was checked by inoculating bacteria on Pikovskya's medium by spot test method followed by measurement of zone ratio¹². Medium possesses insoluble tricalcium phosphate, which on acidification, gives clear zone around colony.

Zinc solubilization

Zinc solubilization efficacy was judged on modified Pikovskaya medium¹³. Pure culture was applied by spot test method and efficacy was judged on the basis of zone of clearance of insoluble zinc oxide substrate.

Siderophore production

Siderophore production was judged by spot inoculation of 1 µl bacterial suspension on chrome azurole S agar plates and incubated at 30 °C temperature for 5 days in dark condition. Appearance of orange halogen around the colony was considered positive test for siderophore production¹⁴.

IAA production

IAA estimation was done using Salkowaskay method¹⁵. A loopful of culture was inoculated in 2 ml Minimal Media amended with 50 µg/ml tryptophan. Inoculated broths were incubated for 24 hrs at 28°C temperature on rotary shaker, centrifuged at 10,000g for 15 minutes, 1ml of supernatant was taken in fresh tube and 2 to 3 drops of Ortho-phosphoric acid was added, 2ml of Salkowaskay reagent (1ml of 0.5M FeCl₃ in 50 ml of

35% HClO₄) was added to the aliquot. Samples were incubated for 25 minutes at room temperature. Absorbance was measured at 530 nm. Auxin quantification values were recorded by preparing calibration curve made by using IAA standard in the range of 10-100 µg/ml.

Identification of Selected Effective Microbes' Bacteria

Total bacterial genomic DNA was extracted and purified according to the CTAB method [16] with slight modifications. The 16S rRNA genes were amplified from genomic DNA with the primers 27F: 5-AGAGTTTGATCCTGGCTCAG-3 and 1492R: 5-CGGTACCTTGTTACGACTT-3. PCR amplification was performed as follows: initial denaturation 5 min at 94°C, followed by 32 cycles of 94°C for 30s, 30s at 40°C, 90s at 72°C, and final extension for 5 min at 72°C, at the last hold at 4°C. Sequencing of amplified 16S rRNA was done using AB prism Sequencer, 3130 Genetic analyzer (Applied Bio systems) with 4 capillaries. DNA sequence homology searches were performed using the online BLAST search engine in GenBank

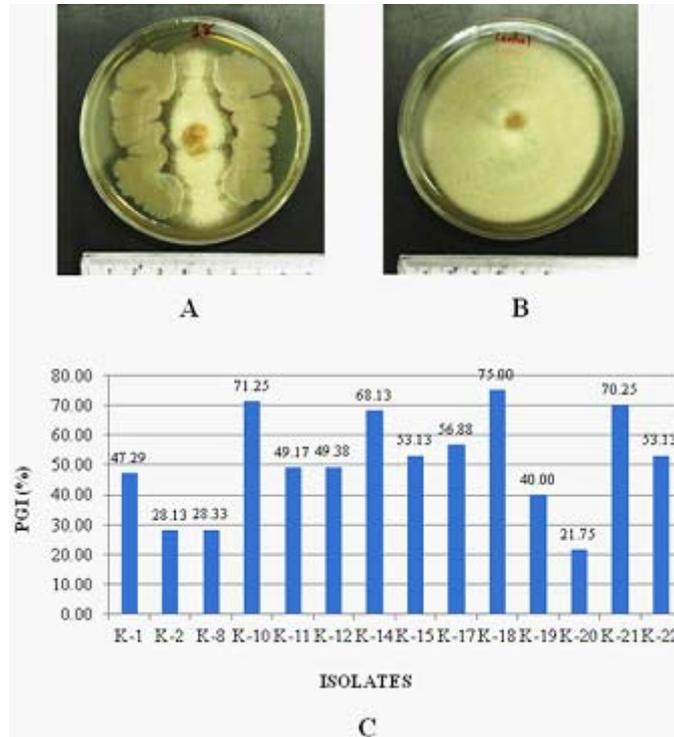


Fig. 1. A. Plate of K18 isolate dual culture after 7 days incubation; B. Control plate of *F. oxysporum* after 7 days incubation; C. Antagonistic potential of different rhizospheric isolates against *Fusarium oxysporum* f. sp.

(available at: <http://www.ncbi.nlm.nih.gov>). Phylogenetic analyses were conducted by using phylogeny.fr¹⁷.

RESULTS AND DISCUSSION

Antagonistic microbes have the potential to inhibit plant pathogenic microorganisms by different mechanisms in eco-friendly manner. Isolation of such important microorganisms was performed from soil samples collected from healthy chickpea rhizospheric soil. Total fourteen rhizospheric bacteria with distinct colony and morphological characteristics were obtained by dilution plate techniques (Table 1) and preserved for future studies.

Use of fungicides in the plant disease management is not advantageous because of its ill effect on environment and non-targeted organisms¹⁸. Use of antagonistic microbes in plant disease management can be the best alternate of fungicides with eco-friendliness. Antagonistic microbes were screened by dual culture method on PDA agar medium against *Fusarium oxysporum* causing wilt in chickpea, the per cent antagonist range from 21.75 to 75.00%. out of which three isolates viz. K18, K10 and K21 showed higher biocontrol potential which was 75.00, 71.25 and 70.25 per cent, respectively, while the lowest efficacy was reported in isolate K20 (21.75%; Fig. 1).

For the characterization of plant growth promotion characters, isolates were also checked for the efficacy to increase the availability of different nutrients i.e. nitrogen, phosphorus and

zinc (Table 2). The isolate K2 and K17 were found positive for increasing the availability of N₂, PO₄ and Zn, the isolate K12 and K20 were found positive in nitrogen fixation only, while remaining isolates K1, K8, K11, K14, K15, K18 and K21 were able to increase the availability of N₂ and Zn. Moreover isolated K19 and K22 were also found promising for phosphate solubilization in lab medium with potential antagonistic activity against *Fusarium wilt* pathogen of chickpea. Iron is a vital element required by every living organisms as a part of electron transport chain and as a cofactor of different enzymes¹⁹. Siderophore producing bacteria produces compound that can chelate the iron element and thus make it unavailable for plant pathogens in rhizospheric region. Siderophore producing bacteria thus works as a biocontrol agent for plant pathogens. Among the different isolates, the isolate viz. K2, K10, K15 and K17 were found to be siderophore positive. Indole 3 Acetic Acid (IAA) is a plant growth promoter and produced by many rhizospheric bacteria, however, none of 14 antagonistic bacteria were found to produce IAA in lab medium amended with tryptophan (Table 3).

The 16S rRNA sequencing of seven potential antagonist bacterial isolates was done for identification. The PCR products of approximately 1380 and 1270 bp were generated for the seven effective antagonistic bacteria. The partial 16S rDNA sequences of all isolates were determined and aligned to other known sequences database in GenBank for their identification (Table 4). The three effective antagonist K10, K18 and K21 were identified as *Burkholderia* sp., *Bacillus subtilis* and *Bacillus aryabhattai*, respectively. The phylogenetic analysis of all seven antagonistic microorganisms using 16s rRNA sequence revealed maximum similarity between isolate K18 and K21 (Figure 2).

The antagonistic effects of twelve rhizosphere isolates (six *Bacillus subtilis* and six *Pseudomonas* spp.) against *Fusarium oxysporum* f. sp. *ciceris* were studied under *in vitro* condition, and found *P. aeuroginosa* (P10 and P12), *B. subtilis* (B1, B6, B28 and B99) and *P. aeuroginosa* (P12 and B28) as a potential biocontrol agent²⁰. The suppression of chickpea *Fusarium* wilt by *Bacillus subtilis* and *Trichoderma harzianum* treatments in liquid and seed inoculation methods was

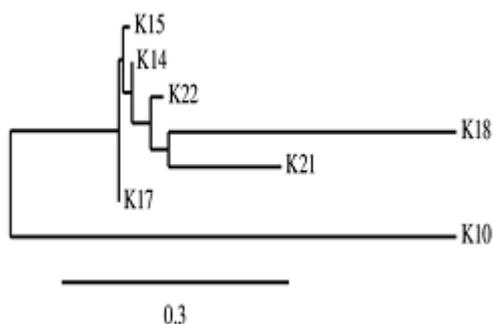


Fig. 2. Phylogenetic relationship among seven highly efficient antagonistic isolated based on 16s rRNA sequence

Table 1. Colony and morphological characters of potential rhizospheric isolates

Sr. no.	Culture ID	Colony characters				Morphological characters						
		Size	Elevation	Shape	Margin	Opacity	Consistency	Pigmentation	Texture	Grams reaction	Shape	Arrangement
1	K 1	Medium	Raised	Round	Entire	Translucent	Sticky	White	Smooth	Negative	Short Rod	Single
2	K 2	Medium	Raised	Round	Entire	Translucent	Sticky	Red	Smooth	Positive	Cocci	Single
3	K 8	Medium	Raised	Round	Entire	Translucent	Sticky	White	Smooth	Positive	Long Rod	Pair
4	K 10	Big	Slightly Raised	Irregular	Entire	Transparent	Sticky	Colourless	Smooth	Negative	Short Rod	Single
5	K 11	Medium	Flat	Oblong	Entire	Translucent	Rough	White	Rough	Positive	Long Rod	Chain
6	K 12	Medium	Flat	Irregular	Undulate	Translucent	Sticky	White	Smooth	Positive	Long Rod	Single
7	K 14	Medium	Raised	Round	Entire	Transparent	Sticky	Colourless	Smooth	Negative	Short Rod	Single
8	K 15	Small	Raised	Irregular	Irregular	Translucent	Rough	White	Rough	Positive	Long Rod	Single
9	K 17	Small	Raised	Irregular	Irregular	Translucent	Rough	White	Rough	Positive	Long Rod	Chain
10	K 18	Big	Flat	Irregular	Entire	Translucent	Rough	White	Rough	Positive	Long Rod	Pair
11	K 19	Medium	Raised	Round	Entire	Translucent	Sticky	Pinkish	Smooth	Positive	Long Rod	Pair
12	K 20	Small	Slightly raised	Irregular	Entire	Translucent	Sticky	White	Rough	Positive	Long Rod	Pair
13	K 21	Small	Raised	Round	Irregular	Translucent	Sticky	White	Sticky	Positive	Long Rod	Single
14	K 22	Big	Flat	Round	Entire	Translucent	Rough	White	Rough	Positive	Long Rod	Single

reported²¹. The antagonistic effect of eight antagonistic microorganisms' viz., *Aspergillus flavus*, *Aspergillus niger*, *Aspergillus ochraceus*, *Azotobacter* sp., *Penicillium* sp., *Pseudomonas fluorescens*, *Rhizobium* sp. and *Trichoderma harzianum* was determined under *in vitro* condition against *Fusarium oxysporium* f. sp. *ciceris*²². All the antagonists reduced the growth of *Fusarium oxysporium* f. sp. *ciceris* significantly but *Trichoderma harzianum* produced longer

inhibition zone (6.72 cm) as compared to other antagonistic organisms. The effects of different antagonists, i.e. *Acrophialophora* sp., *fluorescent pseudomonas* isolates 1, 3, 4 and *Gliocladium virens*, *G. catanaltum*, *G. deliquences*, *Trichoderma hamatam*, *T. harzianum* and *T. koningii* was screened against the chickpea wilt pathogen, and found that *T. hamatam* inhibited an average of 26.03 per cent of radial growth, followed by *fluorescent pseudomonas*-3 (25.5%) and

Table 2. Efficacy of different isolates for increasing availability of nutrients

Sr. no.	Culture ID	Nitrogen fixation	Phosphate solubilization	Zinc solubilization
1	K 1	Positive	Negative	Positive
2	K 2	Positive	Positive	Positive
3	K 8	Positive	Negative	Positive
4	K 10	Negative	Negative	Negative
5	K 11	Positive	Negative	Positive
6	K 12	Positive	Negative	Negative
7	K 14	Positive	Negative	Positive
8	K 15	Positive	Negative	Positive
9	K 17	Positive	Positive	Positive
10	K 18	Positive	Negative	Positive
11	K 19	Negative	Positive	Negative
12	K 20	Positive	Negative	Negative
13	K 21	Positive	Negative	Positive
14	K 22	Negative	Positive	Negative

Table 3. Efficacy of different isolates for Siderophore and IAA production

Sr. no.	Culture ID	Siderophore production	IAA production
1	K 1	Negative	Negative
2	K 2	Positive	Negative
3	K 8	Negative	Negative
4	K 10	Positive	Negative
5	K 11	Negative	Negative
6	K 12	Negative	Negative
7	K 14	Negative	Negative
8	K 15	Positive	Negative
9	K 17	Positive	Negative
10	K 18	Negative	Negative
11	K 19	Negative	Negative
12	K 20	Negative	Negative
13	K 21	Negative	Negative
14	K 22	Negative	Negative

Table 4. Identification of potential antagonistic microorganism using 16S rRNA sequence

Culture ID	Organism identified by BLAST	Query Coverage (%)	E Value	Accession no.
K10	Burkholderia sp. SBH-11 gene for 16S ribosomal RNA, partial sequence	74	6e-50	AB366333.1
K14	Bacillus subtilis strain MML2483 16S ribosomal RNA gene, partial sequence	98	0.0	KJ655546.1
K15	Bacillus subtilis strain MML2483 16S ribosomal RNA gene, partial sequence	97	0.0	KJ655546.1
K17	Bacillus subtilis strain OTPB4 16S ribosomal RNA gene, partial sequence	97	0.0	KT265082.1
K18	Bacillus subtilis strain I55 16S ribosomal RNA gene, partial sequence	64	2e-39	KF318812.1
K21	Bacillus aryabhatai strain JPR39 16S ribosomal RNA gene, partial sequence	97	0.0	KR045599.1
K22	Bacillus sp. RA23 16S ribosomal RNA gene, partial sequence	98	0.0	FJ898306.1

fluorescent pseudomonas isolate-4 (25.08%), respectively²³. The antagonistic activity of 21 isolates of *Rhizobium* under both *in vitro* in dual culture and *in vivo* in green house and field conditions was studied against *Fusarium oxysporum* f. sp. *ciceris* race 0. In dual culture study reported that 14 isolates inhibited the mycelia growth of pathogen more than 30% and most effective isolated were Rh8, Rh11, Rh16 and PchSOM, which inhibited fungal growth more than 50 per cent²⁴. In present study, fourteen isolates were finalized based on antagonistic efficacy and distinguishes, of which only three isolates were found to be potential biocontrol agent under *in vitro* study, they also showed potential PGPR activities.

CONCLUSION

Antagonistic microorganisms can be useful for the eco-friendly plant disease management in sustainable agriculture. Apart from antagonistic activity many biocontrol agents also posses PGPR activities, which exert plant growth promotion by direct or indirect mechanisms. In present laboratory study, isolates K17, K18, K21 and K22 obtained from different rhizospheric soil samples were potent in terms of biocontrol and plant growth promotion potential. A combination of these isolates may have biocontrol as well as growth promotion activities like N₂ fixation, Zn solubilization, PO₄ solubilization and siderophore production. However, potential isolates need to be characterized in field condition for mass exploration.

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