

Research Article

Characterization of multi-trait plant growth promoting *Pseudomonas aeruginosa* from chickpea (*Cicer arietinum*) rhizosphere

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Abstract

In the current perusal, 12 isolates of *Pseudomonas* were segregated by rhizospheric soil of chickpea (*Cicer arietinum*) of Madhya Pradesh, India. Isolated test organisms were characterized morphologically, biochemically and 16S rRNA gene sequencing. Out of 12, one isolate designated as P4 was identified as *Pseudomonas aeruginosa* through 16S rRNA gene sequencing, which revealed 100% homology with the strains DSM 50071 and NBRC 12689. The phylogenetic examination was accomplished utilizing MEGA-X to confirm the identity of isolate P4. The nucleotide hierarchy of the 16S rRNA gene from P4 isolate was submitted in the National Center for Biotechnology Information (NCBI) database under gene bank with accession number MT116414. The P4 isolate exhibited multiple plant growth promotion properties like phosphate solubility, indole acetic acid (IAA) production, 1-aminocyclopropane-1-carboxylate (ACC) deaminase, siderophores, ammonia (NH₃) and hydrogen cyanide (HCN) activities, and biocontrol activities against phytopathogenic fungi *Fusarium oxysporum* and *Macrophomina phaseolina*.

Keywords: Aminocyclopropane carboxylic acid (ACC), Ammonia (NH₃), *Cicer arietinum*, Hydrogen cyanide (HCN), Indole acetic acid (IAA), 16S rRNA gene

INTRODUCTION

Chickpea (*Cicer arietinum* L.) has a place with the group of *Leguminosae*, is the third most significant pulse crop in India (Lev-Yadun *et al.*, 2000; FAOSTAT 2011) and over 20% of world yield (Anonymous, 2014). The optimum environment for total chickpeas growth and development is 18 to 26°C in light and 21 to 29°C in the dark (Duke, 1981; Muchlbauer *et al.*, 1988). It's a good wellspring of energy, protein, minerals, nutrients, fiber, and furthermore, contains potentially advantageous wellbeing phytochemicals (Wood and Grusak, 2007). As a common wellspring of starch and protein, thus making it more efficient and moderate for the non-industrial nations without conciliation the nourishment quality (Malunga *et al.*, 2014) and symbiotically connected to roots in nodules of the chickpea plant, the rhizobial bacteria fix nitrogen to ammonia in order to be allowed to expand and biologically fix nitrogen (BNF). The PGPR (Plant Growth Promoting Rhizobacteria) is the multifarious set of microbes that may be obtained into the rhizospheric region on root stratum. In the past few decades, a huge parade of microscopic organ-

isms, including species such as *Pseudomonas*, *Azotobacter*, *Azospirillum*, *Burkholderia*, *Bacillus*, *Serratia* and so on, have been accounted for to evolve plant development (Bhattacharyya and Jha, 2012; Chauhan *et al.*, 2015). The PGPR influences growth promotion activity directly linked to seed procurement through hormone growth promotion, bacterial isolation or simplification of the usage of such crop supplements by the environment and indirectly through preventing detrimental impacts of one or more phytopathogens. The appropriate procedure through which rhizobacteria magnifies plant development is not completely unequivocal, yet are thought to comprise, that has the capacity to deliver phytohormones such as indole acetic acid (IAA), ethylene, gibberellic acid, cytokinins, the solubility of phosphates and different supplements, asymbiotic N₂ stabilization, antagonism against phytopathogens, antibiotics and cyanide (Kumar *et al.*, 2012). *Pseudomonas sp.* is the ubiquitous bacteria of industrial significance and common inhabitant into a broad diversity of atmosphere, playing a significant part in plant development advancement, induced systemic resistance, natural control and so on (Yuliar *et al.*,

2015). Among all species of *Pseudomonas*, fluorescent *Pseudomonads sp.* has been read for quite a long time as model biocontrol agents to control plant illness. Hence, in this study, an isolate of *Pseudomonas aeruginosa* (P4), which is believed to be a plant growth promoting microorganism, was obtained from rhizospheric soil of chickpea. The isolate was evaluated for the possession of characteristics like phosphate solubility, phytohormones production like Indole Acetic Acid (IAA), 1-aminocyclopropane-1-carboxylate (ACC) deaminase, siderophore, hydrogen cyanide (HCN), ammonia (NH₃) and antagonism against phytopathogens etc. and molecular characterization based on 16S rRNA gene sequencing.

MATERIALS AND METHODS

Sample collection and isolation

The twelve rhizospheric soil samples with plants were collected through developed chickpea to different regions of Madhya Pradesh, India. Samples were placed in large plastic bags and stored at 4 °C until processing within 24-48 hrs. For the isolation of bacteria, soil suspension was serially diluted to a concentration of 10⁴, 10⁵ and 10⁶ in sterile distilled water and poured on King's B medium (King *et al.*, 1954) and incubation was done at 37 °C for 16-24 hrs for growth. The procedure was repeated 2-3 times to get pure culture. A total of 12 bacterial isolates on the basis of fluorescence/pigmentation on the medium were selected, coded and maintained on the nutrient agar medium (NAM) slants.

Characterization of bacterial isolates:

Morphological and biochemical characterization

Morphological characteristics of 12 bacterial isolates, namely colony structure (colour, cell shape, margination and surface) and cell structure (size, shape and arrangement) were studied. All the 12 isolates were processed through various biochemical tests inclusive Indole, Methyl Red, Voges-Proskauer, Citrate utilization, and H₂S production test etc. and have actively taken part in some enzyme activities like Catalase, Oxidase, and Amylase test and so on according to scientific methods (Harley and Prescott, 2002). The potency of microbes for utilization of several energy sources like carbon used as a substrate was examined by a sugar fermentation experiment.

Molecular characterization:

Extraction of genomic DNA (g-DNA)

To extraction of g-DNA, a single cell was selected by liquid suspension of isolates and were developed into LB medium (Luria-Bertani) (Himedia, India) for overnight on universal shaking incubator at 120 rpm at 37 °C. The g-DNA of bacterial isolates was extracted

through bacterial g-DNA purification kit (HiPura™).

16S rRNA gene amplification

Molecular characterization of isolates was done through 16S rRNA gene sequencing. The g-DNA of isolates were applied for amplification of 16S rRNA gene using 16S rRNA PCR Kit (Semi-Q PCR, Himedia, India), in a 20µl reaction mixture containing 10µl 2X PCR Taq mixture, 2µl Primer mix (10Pmol/µl) (27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'-GGTTACCTTGTGTTACGACTT-3') (Miller *et al.*, 2013)), 1µl Template DNA (up to 50ng of extracted DNA) and molecular biology grade water. DNA samples were amplified on PCR thermal cycler (Applied Biosystems, India).

According to PCR conditions, the mixture was heated at 94 °C for 5 min and subjected to 30 cycles at 94 °C for 30 sec, 56 °C for 30 sec, and 72 °C for 45 sec, followed by a final incubation at 72 °C for 5 min. Then aliquot of 2-3µl of PCR products were electrophoresed on a 1% agarose gel inclusive of ethidium bromide and imaged to DNA bands under UV light into gel documentation system (C200, Azure Biosystem, USA).

Sequencing of PCR product and phylogenetic analysis

1.5kb size of resulting amplicon was purified using DNA purification kit (ExoSAP-IT™ PCR product cleanup reagent, Thermo Fisher) and DNA sequencing was performed at Genombio Technologies Pvt. Ltd. (Pune, India). The nucleotide sequences obtained after DNA sequencing were compared against Genbank database using NCBI BLAST (<http://www.ncbi.nlm.nih.gov/BLAST>). A phylogenetic examination was done by utilizing MEGA-X software (Tamura *et al.*, 2013) and alignment utilizing CLUSTAL-ω (Thompson *et al.*, 1994). The pair wise evolutionary distance was constructed by the Tamura 3-parameter method (Tamura *et al.*, 2013).

Screening of isolates for multiple PGP traits:

Nitrogen fixation test

Isolates were tried for their capability of stabilizing nitrogen utilizing the semisolid Jensen's nitrogen-free medium (JNFB medium) (Dobereiner *et al.*, 1995). 50µl of isolates were inoculated into 5ml JNFB semisolid medium at 28 °C for 48 hrs. After incubation, the development of veil-like pellicles was viewed as possible of nitrogen fixation.

Phosphate solubility test

The usage of tricalcium phosphate as insoluble phosphate was tested for phosphate solubility (Mehta and Nautiyal, 2001). A point inoculum of bacteria was put on the center of the Pikovskaya's medium and then

incubated for 48-72 hrs at 37°C or for 5 days at 27±2°C. After incubation, the plates were checked for the presence of a clear yellow coloured halo formed near to colony.

Indole acetic acid (IAA) production test

Auxin confirmation was tested in yeast extract broth medium containing tryptone and isolates were grown into 50ml broth medium supplemented with 50mg/L of L-tryptophan and 2% NaCl followed by incubation done on an orbital shaker with 200 rpm at 27±2°C for 72 hrs. Thereafter, isolates were centrifuged at 10,000 rpm for 10 min. at 4°C. The amount of IAA production in each isolates was determined by the using of salkowasky's reagent method, in which 1ml of supernatant liquid was assorted with 1ml of salkowasky's reagent (49 ml, 70% Perchloric acid and 2ml, 1M FeCl₃ solution) and incubated for 30 min in the dark at 28±2°C for the development of pink colour, and then absorbance was estimated on UV-Vis Spectrophotometer (UV 1800, Shimadzu) at 536 nm. The amount of IAA produced was scheduled from scientific standard graph of pure IAA (Sarwar and Kremer, 1995).

Ammonia (NH₃) production test

Cultures were examined for ammonia creation into peptone water. Recently developed cultures were inoculated in 10ml of peptone water, and incubation was done at 30°C for 48-72 hrs for 5 days. Nessler's reagent (0.5ml) was included in each tube and produced brown coloured to yellow was shown as a conclusive experiment for NH₃ creation (Cappucino and Sherman, 1992).

Hydrogen cyanide (HCN) production test

For HCN creation, isolates were developed on supplement agar medium (NAM) enriched with 4.4g glycine/l, and then usage of filter paper (Whatman no.1) moistened with 2% sodium carbonate in 0.5% picric acid reagent and set on top of culture petridish. After hermetic with paraffin, incubation was done for 4-5 days at 28°C for development of colour (orange to red) indicated the presence of HCN production.

Siderophore formation test

Siderophore formation was qualitatively assessed by chrome azurol S (CAS) medium (Alexander and Zuberer, 1991). New PGPR cultures were inoculated into CAS medium and incubated in obscurity for 48hrs at 28°C. A yellow-orange colour indicated the presence of siderophores.

ACC deaminase activity test

Isolates were developed into 5 ml nutrient broth for 3-4 days at 30°C during the arrived stationary stage to quantify ACC deaminase activity. To deduce their activ-

ity, cells were resumptive through centrifugation and cleaned two times with 0.1M Tris-HCl (pH 7.5), at that point dissolved into 2ml of modified M9 minimal medium enhanced with ACC at a definite concentration of 5mM and incubation done at 30°C with shaking for another 36-40 hrs then checking the concentration of α-Ketobutyrate at 540nm. The activity of ACC was determined by estimating the formation of α-Ketobutyrate (range b/w 0.1-1.0nmol), produced by the cleavage of ACC through ACC deaminase (Honma and Shimomura, 1978). The concentration of α-Ketobutyrate into each sample was determined through similitude with a scientific graph of α-Ketobutyrate.

Screening for antagonistic effects

Antagonistic activity of all 12 isolates was checked against isolated phytopathogenic fungi *F. oxysporum* and *M. phaseolina* from diseased plant of *Cicer arietinum* usage of dual culture examination as mentioned by Naureen *et al.*, (2009) and Hassan *et al.*, (2010) and also compared against standard fungal cultures of *F. oxysporum* (7693) and *M. phaseolina* (6630) obtained from Indian type culture collection (ITCC), Delhi. Percentage mycelium development prohibition was determine through;

$PGI (\%) = (\text{Diameter of control fungus} - \text{Diameter of test fungus}) \times 100 / \text{Diameter of control fungus} \dots \text{Eq. 1}$

After 7-14 days, the antagonistic effects of isolates against fungal pathogens were checked by observing the mycelial growth by slide culture technique. Briefly, the fungal mycelium from control and treated plates were placed on a slide followed by lactophenol cotton blue flooding, washing and observation under the microscope (Olympus CH-20i/ CX21i) at 10X magnification.

RESULTS

Isolation and characterization of isolates

The cultural, morphological and biochemical characteristics of all the 12 isolates recovered from the rhizosphere of chickpea from different regions of Madhya Pradesh (Mandla, Maihar, Narsinghpur, Rewa, Satna, and Sidhi) were observed to be as viscid colonies with yellowish green pigmentation, round/irregular, and gram-negative (-ve) rod-shaped. These isolates gave the positive results for some activities like citrate utilization, catalase, amylase, and gelatin liquification whereas, given the negative results for methyl red (MR), voges-proskauer (VP), indole, hydrogen sulphide (H₂S) and urease. On the basis of the sugar fermentation experiment, these isolates utilized glucose, sucrose and fructose etc. The results obtained from P4 isolate (Accession No. MT116414) out of 12 isolates are illustrated in Table 1.

Table 1. Morphological and biochemical characteristics of P4 isolate.

Characteristics	Activity of P4 isolate
Gram Staining	-ve
Shape	Rod shape
Pigmentation	+ve
Indole production	-ve
MR (Methyl Red)	-ve
VP (Voges-Proskauer)	-ve
Catalase	+ve
Starch hydrolysis (Amylase)	+ve
Citrate utilization	+ve
H ₂ S production	-ve
Urease	-ve
Gelatin liquification	+ve
Glucose	+ve
Fructose	+ve
Sucrose	+ve

Identification and phylogenetic analysis

The sequence of 16S rRNA gene determined through sequencing of resulting PCR amplification product, amplified from P4 isolate showed close homology (100%) with other *P. aeruginosa* sp., at NCBI database. On the basis of phylogenetic analysis, P4 isolate showed closest relationship with *P. aeruginosa* type strains DSM 50071 and NBRC 12689 (Fig. 1). 16S rRNA gene sequence from P4 isolate was submitted in gene bank and the accession number of this sequence is MT116414.

Multiple plant growth promoting traits

According to the screening and evolution of all 12 isolates for multiple PGP traits, P4 isolate was given the best results among all 12 isolates. On the basis of clear zones observed around the inoculums of bacteria on Pikovskaya's medium, P4 isolate was observed positive result for phosphate solubility. On the basis of quantification, ACC deaminase activity was observed 0.4nmol. Production of IAA in tryptophan amended me-

Table 2. Screening of P4 isolate for PGP attributes.

Plant Growth Promoting Attributes	Activity of P4 isolate
Phosphate solubilization (mm)	15mm
IAA Production (µg/ml)	0.8µg/ml
ACC deaminase (nmol)	0.4nmol
Siderophore	++
HCN Production	+++
NH ₃ Production	+++
Nitrogen fixation	++

Whereas; ++ = Best, +++ = Excellent

dium resulted in the development of pink color, which was evaluated for its quantitative analysis. IAA production gradually increased with time, reaching the maximum (0.8µg/ml) at 72 hrs of incubation, followed by gradually decline (Fig. 2). P4 isolate was also observed to be positive for siderophore production according to the appearance of orange-yellow colour on CAS medium and positive for nitrogen fixation for its ability to grow on JNFB medium. Similarly, it was also tested positive for HCN and NH₃ production (Table 2).

Antagonistic activity

Among biocontrol potential of all 12 isolates as evaluated by testing their antagonistic activity against selected fungal strains (*F. oxysporum* and *M. phaseolina*), P4 isolate showed the best antagonistic activity and as compared with standard fungal strains of *F. oxysporum* (7693) and *M. phaseolina* (6630) (purchased from ITCC, Delhi) and was found to inhibit fungus growth (Table 3, Fig. 3). Further, the antagonistic activity testing showed the growth of fungal mycelial hyphae upon observation on a microscopic slide under a microscope for control samples. But, complete loss of mycelial growth or spores was observed as the best antagonistic activity of P4 isolate against *F. oxysporum* and *M. phaseolina* (Fig. 4).

DISCUSSION

The microbial communities present in rhizospheric soil assume a huge part in crop production, soil texture and soil wellbeing. Pulses are referred to develop soil wellbeing further as they harbour favourable microorganisms in their rhizosphere. Chickpea is a significant crop of India and its production is most elevated among all pulses (Perez-Montano *et al.*, 2014).

In the present study, *Pseudomonas* isolates were mostly found in rhizospheric soil of chickpea and were evaluated for their capability to be utilized for sustainable agriculture as a potential biocontrol and plant growth promoting agent.

Phosphate solubilizing bacteria (PSB) are considered as the potential agent for changing unavailable inorganic and organic aspects of phosphorus into plant accessible form. Hence, phosphate solubilization is another significant trait of PGPR and the isolates of current perusal declared prominent liberation of PO₄³⁻ from inorganic phosphate complex, Tricalcium phosphate. Therefore, the present study's conclusion concur with various published literature revealing solubilization of phosphate by *Pseudomonas*, *Bacillus*, and *Azotobacter* (Ahemad and Khan, 2010; Panhwar *et al.*, 2014).

In the present experiment, among all 12 isolates, P4 isolate was identified as *Pseudomonas aeruginosa* and was screened as good phosphate solubilizing bacteria (Table 2). It showed 15mm halo zone on Pikovskaya's

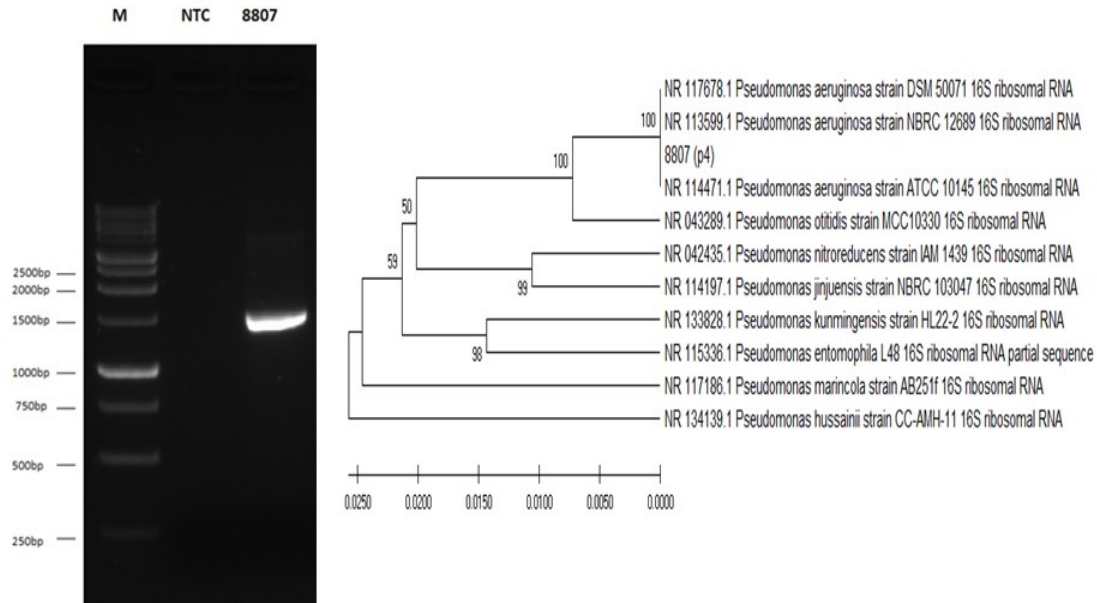


Fig. 1. 16S rRNA gene amplification of P4 isolate and its evolutionary analyses were conducted in MEGA-X.

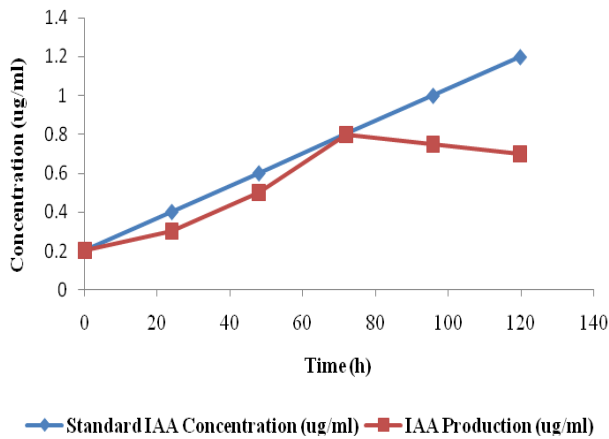


Fig. 2. Production of IAA by P4 isolate at different intervals.

agar plate after 5-6 days of incubation. Multifarious groups of fluorescent *Pseudomonas* like *P. fluorescens*, *P. aeruginosa*, and another species are considered good solubilizers of phosphate that generate radiance of more than 15mm diameter near microbial colonies (Ahmad *et al.*, 2008; Verma *et al.*, 2010; Panhwar *et al.*, 2012).

Indole acetic acid (IAA) is quite possibly the main plant development hormones and it has been reported that it is synthesized in 80% of rhizospheric microorganisms of crops which thus advance root extension and lateral root formation and further develop supplement take-up proficiency of plants (Patten and Glick, 2002). The microorganisms promote plant development by plant chemicals which in turn increase the production of plant metabolites which can be advantageous for the development of the microorganisms (Egamberdieva *et al.*, 2017).

In the present experiment, IAA production was highest in an isolate of P4 (Fig. 2 and Table 2). According to

scientists, the IAA production is recorded in *Pseudomonas* sp., *Bacillus*, *Azotobacter*, and *Rhizobium*, in which IAA production alters with microbes and concentration of tryptophan. Even though the natural efficiency of microbes can produce IAA into the rhizosphere, which only depends upon the utility of precursor of molecules and uptake of bacterial IAA through plant (Ahmad *et al.*, 2008; Verma *et al.*, 2010; Panhwar *et al.*, 2012).

The production of siderophore, ammonia (NH₃) and hydrogen cyanide (HCN) is another attribute that involves indirect traits of plant development, elevating microorganisms to elevate the development of plants. Siderophores are the low molecular weight compounds produced by certain soil microorganisms that develop iron supplement accessibility to plants. In an aerobic environment, iron gets mainly in Fe³⁺ forms hydroxides and oxy-hydroxides and renders itself open to both plants and organisms (Khan *et al.*, 2006). In the present experiment, P4 isolate was also found to be positive for siderophore production (Table 2) and there are likewise a few reports describing the production of siderophores by the rhizospheric microorganisms increasing the iron take-up of plants (Anitha and Kumudini, 2014; Kotasthane *et al.*, 2017).

The production of ammonia and hydrogen cyanide by microorganisms helps the plants both directly and indirectly. In the present experiment, the P4 isolate was able to produce more amounts of NH₃ and HCN (Table 2). The ammonia excreted by diazotrophic microorganisms is possibly the main character of the PGPRs, which advantage the crop. This accumulation of ammonia in soil may enhance the soil pH, becoming aversive to the development of certain pathogenic microorganisms. It likewise disturbs the equilibrium of microbes and inhibits germination of spores of numerous growths

Table 3. Antagonistic activity of P4 isolate against isolated and standard fungal strains.

Test fungi	Colony Diameter (in mm)	Percent (%) inhibition (in mm)
Isolated fungal strains		
Control	75mm	0%
<i>Fusarium oxysporum</i>	25mm	66.7%
Control	80mm	0%
<i>Macrophomina phaseolina</i>	21mm	73.8%
Standard fungal strains		
Control	70mm	0%
<i>Fusarium oxysporum</i> (7693)	20mm	71.4%
Control	70mm	0%
<i>Macrophomina phaseolina</i> (6630)	26mm	62.8%

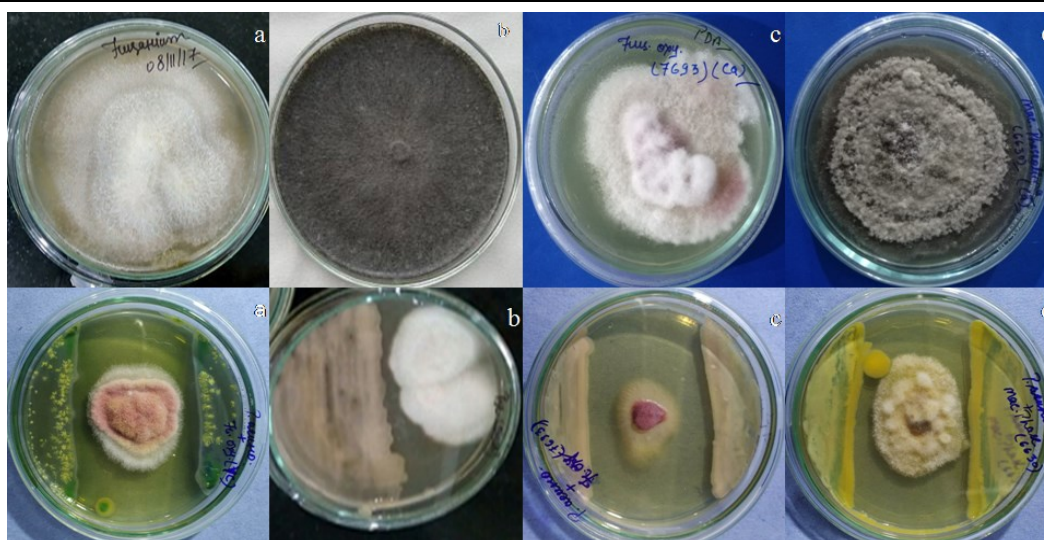


Fig. 3. Inhibition of fungal pathogens *F. oxysporum* and *M. phaseolina* by P4 isolate, (a) Control (isolated *F. oxysporum*) and (a) Test (P4 with *F. oxysporum*); (b) Control (isolated *M. phaseolina*) and (b) Test (P4 with *M. phaseolina*); (c) Control (standard *F. oxysporum*, 7693) and (c) Test (P4 with 7693); (d) Control (standard *M. phaseolina*, 6630) and (d) Test (P4 isolate with 6630)

(Arumugam *et al.*, 2017).

Microorganisms capable of lowering levels of 1-aminocyclopropane-1-carboxylate (ACC), which is the biosynthetic forbear by phytohormone ethylene, may magnify more root development (Glick 2005; Saleem *et al.*, 2007). In the present work, the development of ACC as a nitrogen source indicated that the formation of ACC deaminase was a lot more efficient in the P4 isolate (Table 2). This is certainly seen with *P. fluorescens* CHA0 changed with ACC deaminase gene (Wang *et al.*, 2000). ACC deaminase is liable for dissociation of stress-induced ACC into ammonia and α -Ketobutyrate, which in any case forwarded to produce ethylene that radically affects physiology, development and advancement of plants (Barnawal *et al.*, 2014; Heydarian *et al.*, 2016; Ali and Kim, 2018; Saleem *et al.*, 2018; Zhang *et al.*, 2018).

The antagonistic activity of P4 isolate was observed to be best into controlling the plant root microbes' inclusive of *F. oxysporum* and *M. phaseolina* (Table 3 and Fig. 3 and 4). Several studies have proved that lytic

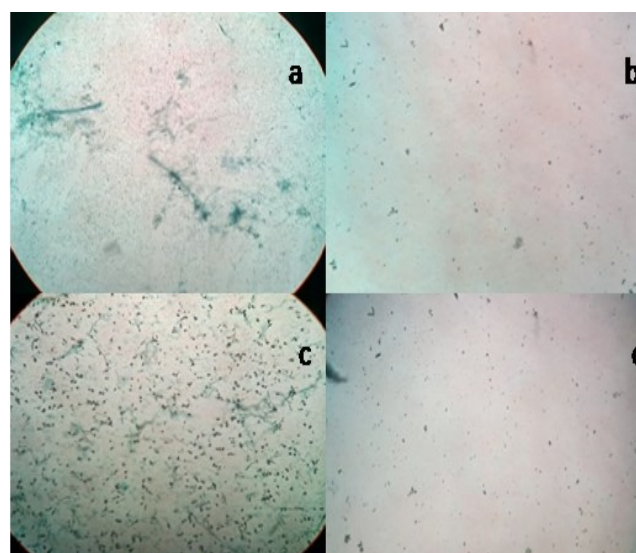


Fig. 4. Microscopic view of fungal isolates antagonistically treated with P4 isolate, (a) *F. oxysporum*; (b) antagonistic effect of P4 isolate against *F. oxysporum*; (c) *M. Phaseolina*; (d) antagonistic effect of P4 isolate against *M. phaseolina* at 10X

catalysts production through *Pseudomonas* strains was influential in detaining plant root microbes' including *F. oxysporum* sp. and *M. phaseolina*, and others (Pal *et al.*, 2001; Shekhar, 2002; Ahmad *et al.*, 2008; Araujo and Guerreiro, 2010).

Further studies based on the representation of P4 isolate upon development and advancement of chickpea will reveal the capability of its utilization as a biocontrol agent and best plant development advancing microorganism.

Conclusion

The present study concluded that among 12 isolates screened for the PGP attributes, *P. aeruginosa* (P4) isolate represented better PGP attributes and antagonism against *F. oxysporum* and *M. phaseolina*. Thus, based on the results, *P. aeruginosa* (P4) isolate can be used as a biocontrol agent and PGP bacteria for the better production and yield of chickpea.

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Conflict of interest

The authors declare that they have no conflict of interest.

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