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Research Article

Exploitation of indigenous fluorescent pseudomonads for the management of wilt of chickpea caused by *Fusarium oxysporum* f. sp. *ciceris*

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ABSTRACT: Chickpea wilt caused by *Fusarium oxysporum* f. sp. *ciceris* is a devastating soil-borne disease with a significant impact on yields and affecting chickpea production worldwide. Fluorescent pseudomonads are utilized as effective biocontrol agents (BCA) against a variety of phytopathogens and they play a key role in pathogen suppression through various processes. In the present study, twenty indigenous fluorescent pseudomonads were isolated from twenty soil samples collected from different districts of North Eastern Karnataka. The isolates were tested *in vitro* for their ability to resist the pathogen by using a dual culture approach. Further, morphological and biochemical characters were studied by growing these isolates on King's B agar medium. All twenty isolates showed inhibition of the test pathogen, with isolate PF-19 showing the highest inhibition of 88.89%. All the isolates developed slimy, irregular colonies with light yellowish green pigmentation, fluorescence under UV light and rod-shaped cells under the microscope, as well as gram negative in reaction. All isolates except PF-2, Pf-9 and PF-10 revealed positive results for KOH, catalase, gelatin liquefaction and starch hydrolysis.

KEYWORDS: Antagonistic potential, chickpea, fluorescent pseudomonads, *Fusarium ciceris*

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INTRODUCTION

Chickpea, an annual legume crop is the world's second most important pulse crop after the common bean. Chickpea is susceptible to a variety of biotic and abiotic stressors that can be found throughout the country. Diseases are the most important biotic limitations to chickpea productivity, causing yield losses of up to 100%. The wilt is the most deadly and prevalent chickpea disease in the world's chickpea-growing regions caused by *F. oxysporum* f. sp. *ciceris* (Trapero-Casas & Jimenez-Diaz, 1985). The fungus is a soil-borne pathogen, although there are few reports that it is also a seed-borne saprophyte. In the absence of a vulnerable host, it can live in the soil for up to six years (Haware *et al*., 1978). Internal xylem discolouration is visible in wilted plants when the roots split open vertically (Nene *et al*., 1991). Early wilting causes more loss than late wilting, while late-wilted plants' seeds are lighter, rougher, and duller than healthy plants' seeds (Haware *et al*., 1978). The entire plant or plant portions may perish within a few weeks as a result of infection. If the variety is vulnerable, the characteristic withering can be noticed 3-4 weeks after sowing in field circumstances.

Biological control of plant infections is an appealing alternative to modern agriculture's heavy reliance on expensive chemical fungicides (Harman *et al*., 2004). Pseudomonas is a Gram-negative bacterium that belongs to the Phylum Proteobacteria, Class -Proteobacteria, and the family *Pseudomonadaceae*, which contains both fluorescent and non-fluorescent pseudomonads. Fluorescent pseudomonads are utilized as effective Biocontrol Agents (BCA) against a variety of phytopathogens, and they play a key role in pathogen suppression through various processes. Because of their propensity to create extracellular secondary metabolites that inhibit the growth of fungal infections and contribute to part of the disease-suppressive effect, they have a lot of potential as BCA. Utilization of these microbial antagonists against plant pathogens in crops has been proposed as an alternative to chemical pesticides (Pal & McSpadden, 2006). Fluorescent pseudomonads are being used as effective BCA against an array of phytopathogens. By considering the above points the present study was carried out to evaluate the rhizospheric antagonistic bacteria against the wilt pathogen *in vitro*.

MATERIALS AND METHODS

The experiments of the present study were conducted at the Department of Plant Pathology, Agricultural College, University of Agricultural Sciences, Raichur during 2020-2021.

Isolation of *Fusarium oxyxporum* f. sp. *ciceris*

Chickpea plants with typical wilt symptoms were taken and sterilized bits of stem were inoculated on Potato Dextrose Agar (PDA) medium and cultured for 5 to 7 days at 28 ± 1°C. The pathogen was purified by using the hyphal tip technique (Rangaswami, 1972). Koch's postulates were proved by using the susceptible variety Annigeri-1.

Molecular identification of the pathogen

Identification of the pathogen was studied by using ITS 1 and ITS 4 primers. Standard protocols were used for the isolation of DNA and ITS analysis according to Dubey *et al.* (2012).

Isolation of fluorescent pseudomonads from chickpea rhizospheric soil

A total of twenty samples were obtained from various taluks in North Eastern Karnataka. The samples were taken to the Bio-input Entrepreneurship Centre, College of Agriculture, University of Agricultural Sciences, Raichur, where fluorescent pseudomonads were isolated. Using King's B medium (KBM), fluorescent pseudomonads were isolated from soil using a serial dilution approach (King's B medium: Ingredients per lit: Peptone: 15 g; $MgSO_4$: 1.5 g; K_2HPO_4: 1.5 g; glycerol: 10 ml; agar: 15 g).

Antagonistic potential of fluorescent pseudomonads against *F. oxysporum* f. sp. *ciceris*

Using a dual culture approach, all the twenty fluorescent pseudomonad isolates were tested for antagonistic capability against the pathogenic fungus *F. oxysporum* f. sp. *ciceris*. Pathogen radial growth was measured with bacterial culture to evaluate the degree of antagonism. The per cent inhibition over the control was calculated by using the formula (Vincent, 1947).

$$I = \frac{(C - T)}{C} \times 100$$

where,

I = Per cent inhibition
C = Radial growth of fungus in control
T = Radial growth of fungus in treatment

All isolates of fluorescent pseudomonads were grouped based on their inhibition performance like I: ≥70%, II: 50-69%, III: 26-49%, IV: ≤25%.

Volatile compounds production by native fluorescent Pseudomonads and their effect on *F. oxysporum* f. sp. *ciceris*

The test for inhibitory volatile compounds production by fluorescent pseudomonads was carried out by inverted plate technique (Dennis and Webster, 1971). Two lids of separate sterilized Petri plates were taken and 20 ml of PDA medium was poured for one plate and 20 ml of King's B medium (KB) on another plate and allowed for solidification. A loopful of 48 h old fluorescent pseudomonads culture was streaked on the plate containing KB medium and mycelial disc (5 mm) of pathogen *F. oxysporum* f. sp. *ciceris* was inoculated at the centre of the plate containing PDA medium. The colony diameter of the pathogen was measured and compared with control. The per cent inhibition was calculated by using the formula of Vincent (1947).

Morphological characterization

Characters such as fluorescence generation under UV light, cell morphology and spore production were investigated and all isolates were grown at 4°C and 36°C. A loopful culture of luminous pseudomonads was streaked on *Pseudomonas* agar media and incubated at 28 ± 2°C for 48 h for pigment development, with the colony examined under UV light for fluorescence generation.

Biochemical characterization

Gram's staining

As per the American Phytopathological Society's Laboratory Guide for Identification of Plant Pathogenic Bacteria, the standard approach for Gram staining (Schaad, 1992) was used for all the isolates of fluorescent pseudomonads.

KOH test

The colony was gently mixed with the use of an inoculation loop by adding 3% KOH. If a mucoid thread forms, the bacteria is Gram negative; if it does not, the bacterium is Gram positive.

Catalase test

The rapid growth of O_2, when H_2O_2 was added, demonstrated by the formation of bubbles, had a good effect on catalase production.

Gelatin liquefaction

The test cultures were injected into pre-sterilized nutritional gelatin deep tubes and cultured for 24 h at 28 ± 2 °C. The tubes were then chilled for 30 min. at 4 °C Tubes with liquefied cultures were regarded positive for the test, while those that hardened after cooling were considered negative (Blazevic & Ederer, 1975).

Starch hydrolysis test

The test was considered positive when a clear zone formed around the colony in a starch-containing medium (Eckford,

1927).

Statistical analysis

The data obtained in the present investigations for various parameters in the experiments were subjected to ANOVA for a completely randomized design (CRD) for *in vitro* studies by using the OPSTAT programme.

RESULTS AND DISCUSSION

Isolation of pathogen *F. oxysporum* f. sp. *ciceris*

At seven days after incubation, a whitish colony of a fungus with fuzzy profuse mycelium was seen. It eventually turned to pink. The pathogen was identified using standard mycological criteria based on mycelial and conidial features. The fungus generated a large number of spindle-shaped, curved macroconidia with three to five septa, whereas microconidia were fusiform with rounded apex and no septa. The chlamydospores were globose to oval in shape, had a thick wall, and appeared terminally or intercalarily (Figure 1). Nelson (1981) and Di-Pietro *et al*. (2003) made similar results, reporting that *F. oxysporum* develops colourless to pale mycelium that turns pink or purple with age when produced in culture with ovoid microconidia and spindle-shaped macroconidia with septa. Chlamydospores with one or two cells might be terminal or intercalary.

Both the primers ITS-1 and ITS-4 produced an amplified product size of 500-550 bp indicating that the isolate belongs to the species *Fusarium oxysporum.* Further, nucleotide sequencing was done the for ITS region of 18S rRNA. The BLAST data revealed that the *Fusarium oxysporum* species matched with the reference strains of NCBI results and identified as *Fusarium oxysporum*.

Isolation of fluorescent pseudomonads

All twenty isolates displayed characteristic fluorescence under UV light after 48 h of incubation. On KBM, the representative colony type was chosen and purified (Figure 2). Joseph *et al*. (2007) also collected six rhizospheric soil samples from chickpea-growing fields and used King's B medium to extract bacterial strains. Navprabhjot and Poonam (2013) isolated 35 *Pseudomonas* spp. isolates from soil samples taken from the chickpea rhizosphere on King's B medium.

Antagonistic potential of fluorescent pseudomonads against *F. oxysporum* f. sp. *ciceris*

The per cent suppression of *F. oxysporum* f. sp. *ciceris* mycelial growth ranged from 16.67 to 88.89%, according to the findings of the antagonism test. Four isolates showed the greatest inhibition (>75%) (PF-7, PF-15, PF-16 and PF-19). PF-19 had the highest percentage inhibition of 88.89%, followed by PF-15 (74.07%) and PF-7 (74.07%). The results are presented in Table 1 and Figure 3. The findings of this study agree with those of Kandoliya and Vakharia (2013), who found that all ten *P. fluorescens* isolates suppressed the growth of *F. oxysporum* f. sp. *ciceris*. At 6 days after inoculation, *P. fluorescens* (Pf-3) showed the highest percentage of growth inhibition (83.50%). Venkataramanamma *et al*. (2019) tested twelve fluorescent *Pseudomonas* isolates against *F. oxysporum* f. sp. *ciceris* and found that the CRP-6 isolate had the highest percent inhibition (58.32%).

Volatile compounds production by native fluorescent pseudomonads and their effect on *F. oxysporum* f. sp. *Ciceris*

The results showed that the isolates produced a considerable amount of volatile compounds which varied within the isolates. A higher concentration of volatile compounds was produced by isolate PF-19 (66.67 %) followed by PF-14 (64.44 %), PF-6 (56.67 %) and a lower concentration of PF-9 (4.44 %) (Table 2, Figure 4). Similar results were also reported by Poonam *et al*. (2013), who studied the effect of volatile metabolites produced by *Pseudomonas fluorescens* on growth inhibition of *F. oxysporum* f. sp. *ciceris* in Ps-17 (13.80 %) and Ps-14 (9.20 %). Further, a higher concentration of volatile metabolites was produced in isolates Pf-2, which inhibited *F. oxysporum* f. sp. *ciceris* by 100 per cent. Pf-15 and Pf-28 were inhibited by 78.20 and 74.40 per cent, respectively (Architha, 2018).

Morphological characterization

After 24 hours of incubation, all of the isolates began to grow. All of the isolates generated slimy, irregular colonies with light yellowish-green pigmentation and UV fluorescence and the cells were rod-shaped under microscopic examination. These traits were taxonomically useful for identification of *Pseudomonas* (Cartwright & Benson, 1985). Furthermore, bacteria grew best at 36°C and did not grow at 4°C. The results are shown in Figure 5.

Under the microscope, Saikia *et al*. (2010) identified twenty-five isolates of fluorescent pseudomonads, all of which were rod-shaped and luminous in the presence of UV light. Nirmala and Reddy (2014) looked at 55 fluorescent *Pseudomonas* isolates, which were rod-shaped and produced fluorescent pigment when exposed to UV light. Under UV illumination, all five isolates examined developed slimy, uneven colonies with yellowish green colouring. Earlier researchers had reported similar findings (Manasa *et al*., 2017; Shruthi, 2017).

Biochemical characterization

All fluorescent pseudomonad isolates showed Gram negative responses and pink-coloured cells when observed

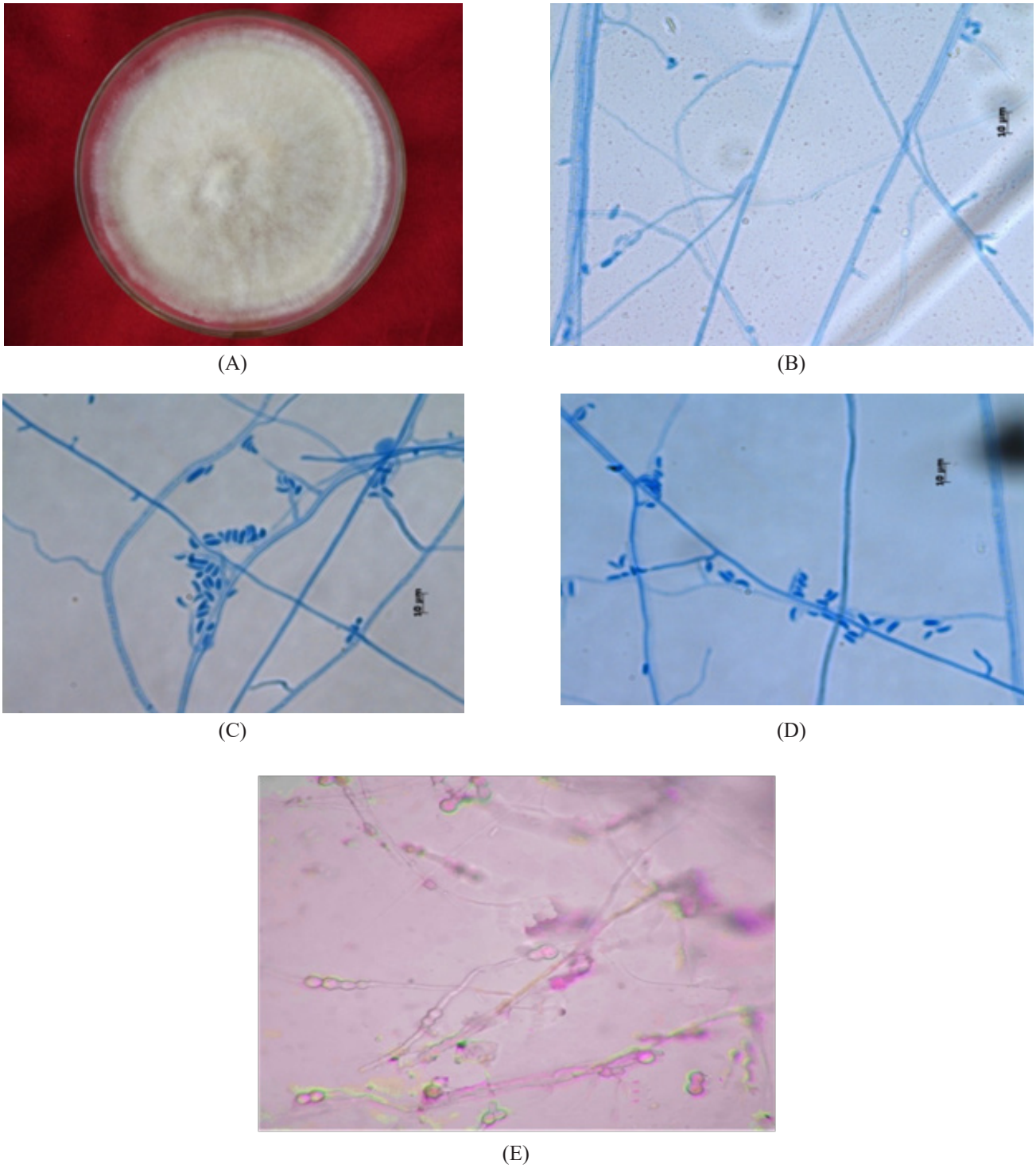


Figure 1. Culture and morphological characters of *F. oxysporum* f. sp. *ciceris* Molecular identification of the pathogen **(A)**. Growth on PDA, **(B)**. Branched septate mycelium, **(C).** Micro conidia, **(D).** Macro conidia, **(E).** Chlamydospores.

Table 1. Antagonistic potential of native fluorescent pseudomonads against *F. oxysporum* f. sp. *ciceris*

Sl. No	Isolate code	Colony growth* (mm)	Per cent mycelial inhibition*	Rank
1	PF-1	40.67	54.81 (47.76)	II
2	PF-2	45.67	49.26 (44.57)	III
3	PF-3	75.00	16.67 (24.09)	IV
4	PF-4	62.33	30.74 (33.67)	III
5	PF-5	49.00	45.56 (42.44)	III
6	PF-6	28.33	68.52 (55.86)	II
7	PF-7	25.00	72.22 (58.19)	I
8	PF-8	50.67	43.70 (41.38)	III
9	PF-9	72.00	20.00 (26.56)	IV
10	PF-10	60.67	32.59 (34.81)	III
11	PF-11	65.00	27.78 (31.80)	III
12	PF-12	44.67	50.37 (45.21)	II
13	PF-13	60.67	32.59 (34.81)	III
14	PF-14	30.00	66.67 (54.73)	II
15	PF-15	23.33	74.07 (59.38)	I
16	PF-16	27.00	70.00 (56.78)	I
17	PF-17	30.00	66.67 (54.73)	II
18	PF-18	51.00	43.33 (41.16)	III
19	PF-19	10.00	88.89 (70.52)	I
20	PF-20	50.00	44.44 (41.80)	III
21	Control	90.00	0.00 (0.00)	-
S. Em. ±		**-**	**0.61**	**-**
CD at 1%		**-**	**2.31**	**-**

*Mean of three replications

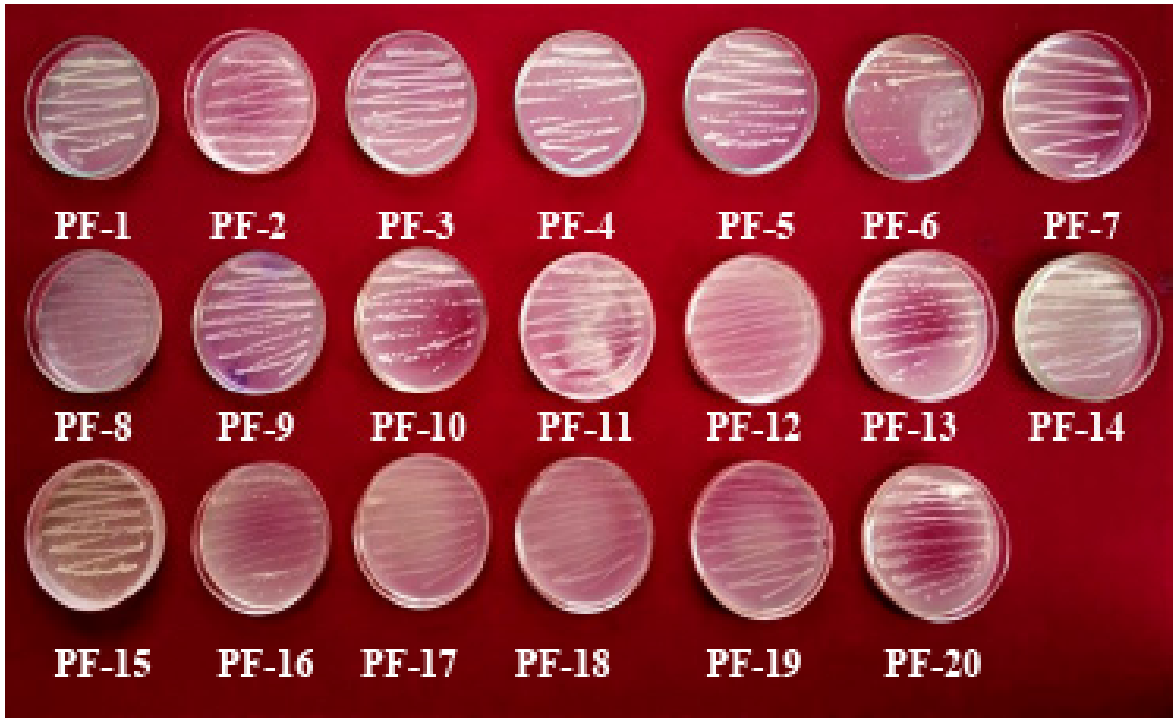


Figure 2. Isolates of fluorescent pseudomonads collected from chickpea rhizosphere.

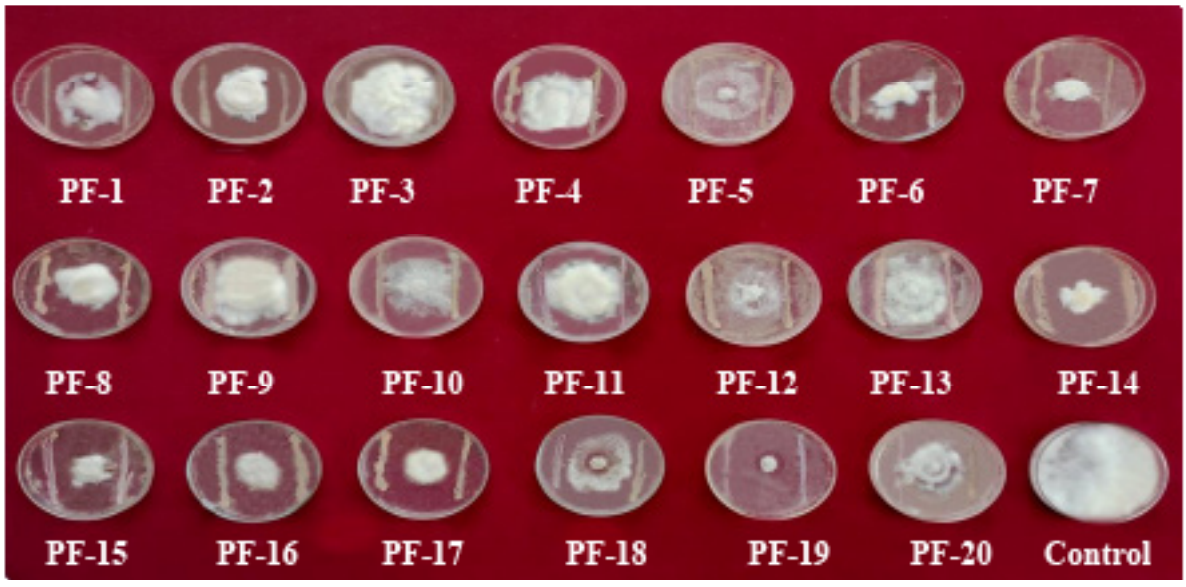


Figure 3. Antagonistic potential of native fluorescent pseudomonads against *F. oxysporum* f. sp. c*iceris.*

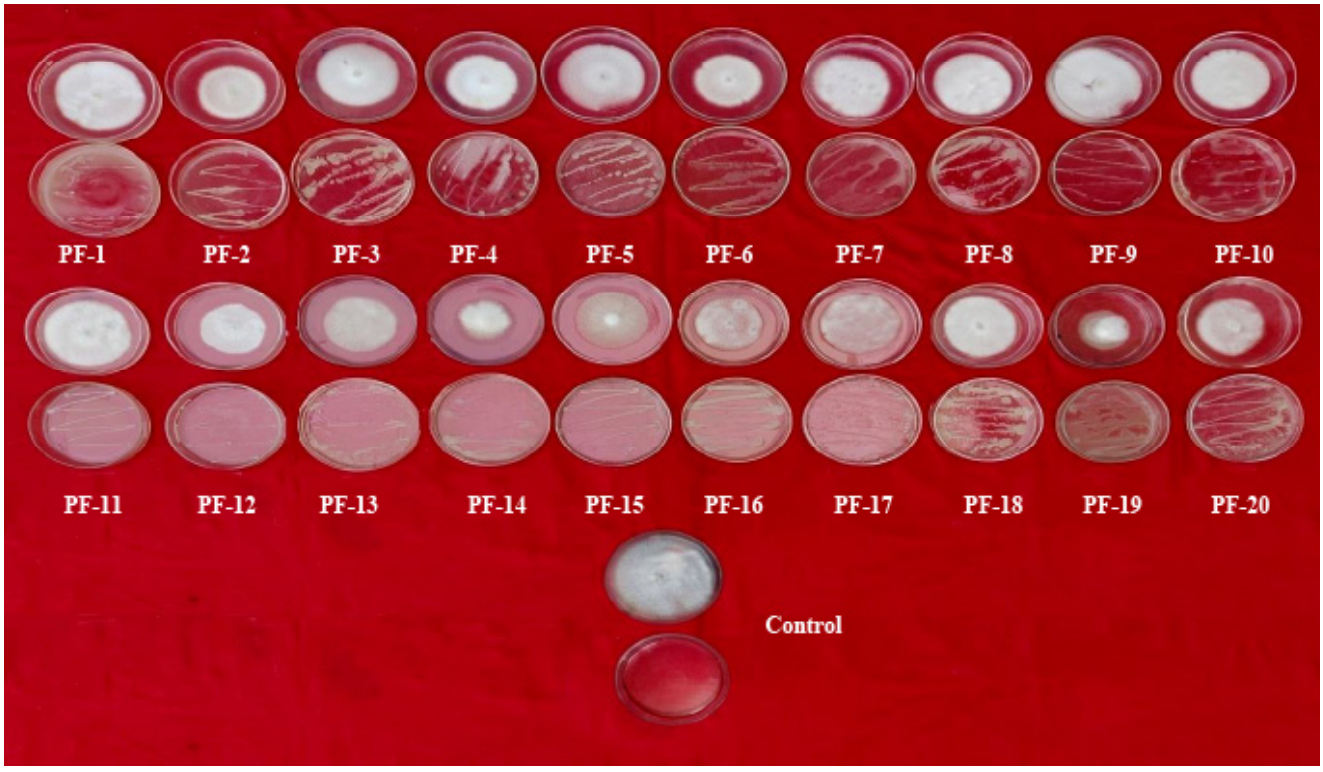


Figure 4. Mycelial inhibition of *F. oxysporum* f. sp. *ciceris* by volatile compounds produced by native fluorescent Pseudomonads.

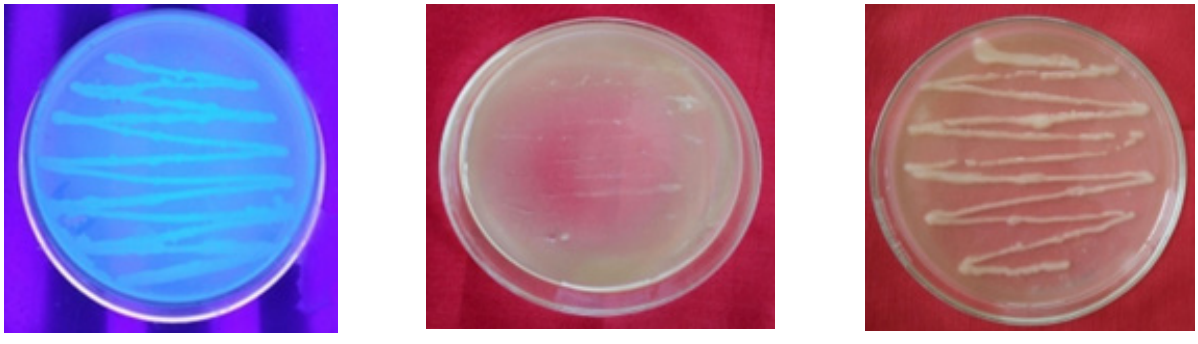
under a microscope. A loop of pure bacteria culture was placed on a glass slide and mixed with 3% KOH. When lifted, it formed a string with the loop and was recorded as a positive test. The test was positive for all twenty isolates in this research. In the catalase test, all of the isolates created gas bubbles, indicating a positive result. Except for PF-2, PF-9 and PF-10, all isolates tested positive for the gelatine liquefaction test. Except for PF-2, PF-9 and PF-10, all isolates showed formation of a clear zone around the starch in the hydrolysis test (Figure 6).

Nirmala and Reddy (2014) made similar observations when they described *P. fluorescens* using biochemical tests as prescribed in Bergey's manual of systematic bacteriology. All of the isolates tested were Gram negative, positive for KOH, catalase, and gelatin liquefaction, but negative for

Table 2. Effect of volatile compounds produced by native fluorescent Pseudomonads on mycelial inhibition of *F. oxysporum* f. sp. *ciceris*

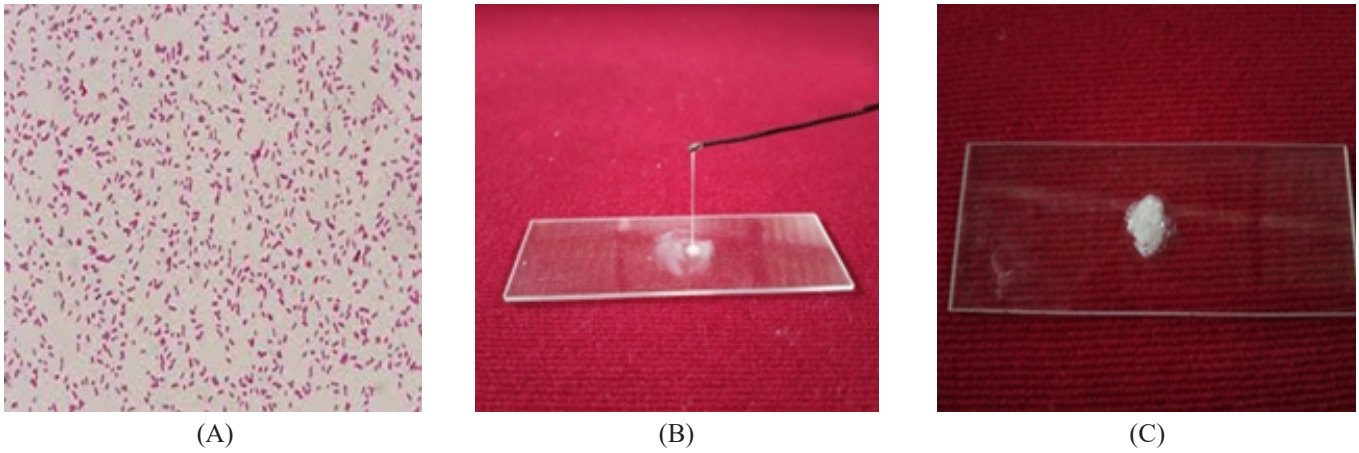
Sl. No	Isolate code	Colony growth* (mm)	Per cent mycelial inhibition*
1	PF-1	60.00	33.33 (35.26)
2	PF-2	40.00	55.56 (48.18)
3	PF-3	60.00	33.33 (35.26)
4	PF-4	54.67	39.26 (38.79)
5	PF-5	75.00	16.67 (24.09)
6	PF-6	39.00	56.67 (48.82)
7	PF-7	54.00	40.00 (39.23)
8	PF-8	56.33	37.41 (37.70)
9	PF-9	86.00	4.44 (12.17)
10	PF-10	63.33	29.63 (32.97)
11	PF-11	64.00	28.89 (32.51)
12	PF-12	44.67	50.37 (45.21)
13	PF-13	50.00	44.44 (41.80)
14	PF-14	32.00	64.44 (53.39)
15	PF-15	45.67	49.26 (44.57)
16	PF-16	54.00	40.00 (39.23)
17	PF-17	75.00	16.67 (24.09)
18	PF-18	65.00	27.78 (31.80)
19	PF-19	30.00	66.67 (54.73)
20	PF-20	70.00	22.22 (28.12)
21	Control	90.00	0.00 (0.00)
S. Em. ±		-	**0.64**
CD at 1%		-	**2.44**

*Mean of three replications, Figures in the parenthesis are arcsine transformed values

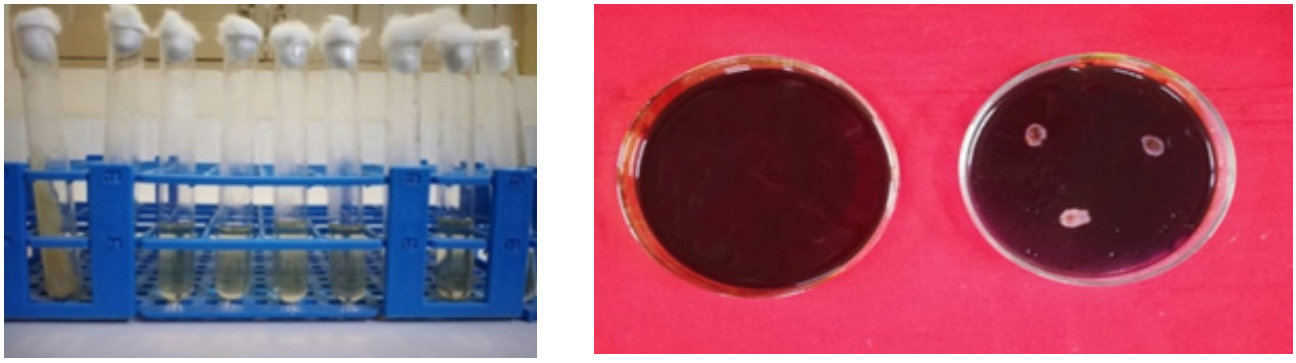


(A) (B) (C)

Figure 5. Morphological characteristics of fluorescent pseudomonads. **(A).** Fluorescence under UV light, **(B)**. Growth at 4ºC, **(C).** Growth at 36ºC.



(A) (B) (C)



(D) (E)

Figure 6. Biochemical characteristics of fluorescent pseudomonads. **(A).** Gram's staining, **(B).** KOH test, **(C)**. Catalase test, **(D)**. Gelatin liquefaction, **(E).** Starch hydrolysis.

starch hydrolysis. Manasa *et al*. (2017) also performed biochemical tests on 15 isolates of *P*. *fluorescens*, including catalase, starch hydrolysis, and gelatin liquefaction. Twelve of the 15 isolates tested positive for starch hydrolysis, only three for gelatin liquefaction, and all were Gram-negative.

CONCLUSION

A total of twenty isolates of fluorescent pseudomonads were shown to have antagonistic activity against *F*. *oxysporum* f. sp. *ciceris,* a wilt causing pathogen of chickpea. All the tested isolates of fluorescent pseudomonads were Gram negative and showed positive results in the case of catalase test, KOH test, gelatin liquefaction test and starch hydrolysis test except PF-2, Pf-9 and PF-10 isolates. The fluorescent pseudomands are efficient tools in the biological control of plant diseases.

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