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Evaluation of different isolates of *Pseudomonas fluorescens* against *Fusarium oxysporum* f. sp. *ciceri*, causing wilt of chickpea (*Cicer arietinum* L.)

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Abstract

Pseudomonas fluorescens is an efficient antagonist against fungal diseases. Many strains of *P. fluorescens* showed their potential towards suppressing the disease severity in chickpeas caused due to *Fusarium oxysporum*. The present study aimed to the evaluation of operative strains isolated from chickpea fields. Out of 20 isolates of *P. fluorescens*, six isolates (Pf4, Pf13, Pf14, Pf18, Pf19, and Pf20) were identified as strong inhibitors against *Fusarium oxysporum* f. sp. *ciceri* (Foc) in dual culture approach, when compared to other bacterial strains of *P. fluorescens*. Later, the inhibitory activity of the same six isolated strains was validated by all cultural and biochemical tests. The antagonistic activity of (Pf 18, Pf 4, Pf 20, Pf 19, Pf13, and Pf 14) was reduced by (80.1, 79.8, 76.4, 73, 72.6, and 70.3) per cent, respectively, as compared to the control. Pf14 isolate out performed other isolates in terms of enhancing shoot and root growth when compared with control. Selected isolates shown showed significant decrease in wilt incidence percentage both in greenhouse and field trials and also increased seed germination (98%) in Pf13.

1. Introduction

Pseudomonas fluorescens has been documented as a complex collection of a large number of described species (Gardener *et al.*, 2005) and is considered an effective growth-promoting Rhizobacteria and biocontrol agent. Many strains of this bacteria are found worldwide in a wide range of environments and show a substantial amount of physiological and genetic flexibility (Nowak-Thompson *et al.*, 1997). The intimate association of many strains with chickpea and inhibitory action against the wilt causing soil born pathogen, *F. oxysporum*, indicate *P. fluorescens* as a functionally and ecologically worthy micro-organism (Salman, 2010; Hebber *et al.*, 1992; Fridlender *et al.*, 1993; Maurya *et al.*, 2020). *P. fluorescens* occurs naturally in the plant rhizosphere and protects the plant by secreting secondary metabolites containing growth-promoting substances, antimicrobial substances, and hydrolytic enzymes such as chitinase and protease (Kohl *et al.*, 2019; Kumari and Khanna, 2019; Agaras *et al.*, 2020).

In recent years, with an increase in population demand for pulses especially chickpeas have been increased in the food and agriculture industry. Because of the high protein content, calcium, iron, phosphorus, and other minerals; chickpea is an important part of a vegetarian's diet (Latham, 1997). The majority of chickpea production and consumption (95 per cent) takes place in underdeveloped countries. According to Kaur *et al.* (2007), infections brought on by fungal, bacterial, nematode, mycoplasma, and viral pathogens have been documented to affect chickpeas. Throughout, 32 countries around the world have reported having the widespread chickpea wilt caused by *F. oxysporum*, and six fungal infections have been identified as being serious and leading to significant crop loss (Haware *et al.*, 1986; John *et al.*, 2022; Kaur *et al.*, 2007). In India, the fungus causes substantial yield losses of 10-15 per cent, which can rise to 60-70 per cent in years of severe epidemics (Jalali *et al.*, 1999).

Chemically synthesized organic and inorganic compound and their indiscriminate use in agriculture have been continued for many years. Chemical treatments for plant diseases are costly while at the same time, biomagnification and toxic consequences of these chemicals have created environmental and public health issues (Shanmugaiah *et al.*, 2015). In addition, chemical-based treatment causes decreased soil productivity, crop quality, and yield loss.

In this scenario, in contemporary agriculture, the use of biological control agents as fungicide alternatives is steadily expanding. *P. fluorescens* can be used to biologically control several plant pathogens, and using plant growth promoting rhizobacteria strains, especially those from the genus *Pseudomonas*, to do so is an effective alternative to using chemical pesticides to control plant diseases, according to several studies (Kumari and Khanna 2019). The exceptional ability of the soil bacterium, *Pseudomonas* to control phytopathogens through a variety of mechanisms, including the production of antibiotics, siderophores, and lytic enzymes, as well as the release

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of volatile antifungal compounds into the atmosphere, appears to be of particular interest to researchers (Shanmugaiah *et al*. 2010; Kumari and Khanna 2019; Karmegham *et al*. 2020). *F. oxysporum* is prevented from growing by the strains *P. luteola* and *P. fluorescens* (Abed *et al*., 2016) and (Rathore *et al*., 2020) reported that *P. fluorescens*-5 inhibited the growth of *F. oxysporum*. According to Kumar *et al*. (1996), under iron-limiting conditions, a *Pseudomonas* strain that produces siderophores inhibits *F. oxysporum* and *F. udum*. Potential *P. fluorescens* strain that could replace the careless application of numerous chemical and inorganic fungicides (Panpatte *et al*., 2016; John *et al*., 2019). The current investigation was done to isolate and assess several isolates of *P. fluorescens* from chickpea fields against *F. oxysporum* to evaluate their antagonistic activity against two isolates of *F. oxysporum* and their capacity to stimulate plant growth (plant growth promoters). The effects of the antagonists on the morphology of *F. oxysporum* isolates were examined under a microscope.

2. Materials and Methods

2.1 Isolation of *Fusarium oxysporum* f. sp. *ciceri*

From the experimental field of the Department of Plant Pathology, SHUATS, Prayagraj, diseased chickpea plants with typical wilt symptoms were collected. On potato dextrose agar (PDA) medium, the pathogens *F. oxysporum* that cause wilt disease in chickpea plants were isolated from the recently infected roots. The roots and stems of recently infected chickpeas were carefully cleaned with distilled water, cut into small pieces (5 mm in length), a small portion of the sick tissues and a small section of the nearby healthy tissues were surface sterilized through 0.1% $HgCl_2$ for 30 sec. The pieces were then cleaned three times with distilled water. The pieces were surface sterilised, washed, and aseptically injected onto a sterile Petri plate with PDA media. The inoculated Petri plates underwent a five to six-day incubation period at 28°C. A small piece (5 mm in length) of a single mycelium was transplanted onto a different Petri plates containing PDA medium once the fungus colony had formed to produce pure culture. By routinely subculturing on fresh medium and keeping them in a refrigerator at 4°C, the pure culture was kept throughout the experiment.

2.2 Isolation of *Pseudomonas fluorescens*

P. fluorescens was isolated from the rhizosphere of chickpea fields in several parts of the Allahabad district. The infected roots were broken into little pieces and thoroughly mixed with the roughly 10 cm of rhizosphere soil that was loosely adhering to them. To create a typical soil suspension, the soil was acquired in this manner and then mashed in a sterile mortar and pestle with 100 ml of sterile distilled water for 10 to 20 min. Using a special King's B medium, the serial dilutions and pour plate approach were used to isolate *P. fluorescens*.

2.3 Pour plate method

King's B medium, a selective medium, was used to isolate *P. fluorescens* (Kings *et al*., 1954). Added 20 ml of sterile medium and 1 ml of soil suspension from aliquot dilutions (10^5 to 10^8) into sterile Petri plates under aseptic conditions and the plates were subsequently incubated for 48 h at 28 ± 2^0C. Using UV light, particular colonies with yellow-green and blue-white pigments were found and identified during incubation. Individual colonies were picked up with sterile loops, transferred to fresh King's B slants, and then placed in refrigerators at 4°C for subsequent usage to create the isolated pure cultures.Several biochemical assays were carried out following Bergey's Manual for Developmental Bacteriology for the identification of *P. fluorescens* (Breed *et al*., 1989).

2.4 Dual culture technique

To identify the most effective strain, the *P. fluorescens* strains (Pf1, Pf2, Pf3, Pf4, Pf5, Pf6, Pf7, Pf8, Pf9, Pf10, Pf11, Pf12, Pf13, Pf14, Pf15, Pf16, Pf17, Pf18, Pf19, and Pf20) were tested in the lab using dual culture techniques on PDA. *Pseudomonas* spp. and *F. oxysporum* were inoculated into Petri dishes (90 mm) containing potato dextrose agar medium at equal distances from the plate's edge. The radial growth of the pathogen, *F. oxysporum* was monitored at intervals of 24 h up to 7 days after incubation on inoculated plates while they were incubated at 25°C in a BOD incubator. Each treatment was repeated three times, and *Pseudomonas* free controls were kept. Seven days after the pathogen and *Pseudomonas* strains were inoculated, observations were made on the affected area. Each strain was evaluated for its ability to prevent the growth of pathogenic fungi's mycelium. The bacterial isolates with the largest zone of inhibition were chosen for additional research.

Arora and Upadhyay (1978) used the following calculation to compute the percentage growth inhibition:

Colony growth in control plate =Colony growth in intersecting plate

$$= \frac{\%\ \text{growth inhibition}}{100}$$

Colony growth in control plate

2.5 Evaluation of antagonistic micro-organism in greenhouse

In order to determine the efficacy of *P. fluorescens* strains in the control of chickpea wilt disease under greenhouse conditions, chickpea seeds were sown in pots containing field soil mixed with *F. oxysporum* cultures cultivated on sand-maize medium.Three pots were kept for each strain and four seeds were planted in each pot. The ratio of the fungal culture to the soil (sand-maize inoculums) was 1:19. Cell suspensions of the chosen *P. fluorescens* strains in water (10^9cfu per ml) were used to treat the seeds. We evaluated the wilt incidence up to 90 days after seeding.

2.6 Evaluation of antagonistic microorganism in field

Three times' worth of the randomized complete block (RBD) design was used to experiment. The sick field had been thoroughly manured and fully prepared. After being infected with various strains of *Pseudomonas*, the seeds of the extremely vulnerable chickpea variety "Uday" were then placed in the shade for 30 min to allow natural drying. Then, three replications of each treatment were used to sow the seeds using a randomized block design. On October 28, 2015, chickpeas were sown in plots measuring 2 × 2 m^2 by a spacing of 30 x 10 cm between rows and plants, respectively. A control plot was kept by only treating the chickpea seeds. Wilt incidence as well as root and shoot expansion were noted.

2.7 Data analysis

For the *in vitro* experiment, the observations were noted and statistically assessed, while for the field experiment, a randomised block design was used (Gomez and Gomez, 1984).

3. Results

3.1 Isolation of *Pseudomonas fluorescens* isolates

Twenty *P. fluorescent* strains, including Pf1, Pf2, Pf3, Pf4, Pf5, Pf6, Pf7, Pf8, Pf9, Pf10, Pf11, Pf12, Pf13, Pf14, Pf15, Pf16, Pf17, Pf18, Pf19, and Pf20, were tested *in vitro* for antagonistic activity against *F. oxysporum* in PDA. The results demonstrated that all of the antagonistic *Pseudomonas* strains utilised in the current investigation strongly inhibited the growth of *F. oxysporum* mycelia. Six strains were identified as having the significantly largest mycelial growth inhibition against pathogens out of all of these antagonistic strains. They were chosen for more research.

3.2 Dual culture technique

Each antagonist isolates significantly inhibited the development of the test fungus, according to dual culture testing. Pf18 demonstrated the greatest (80.1%) and Pf7 the least (57.2%) mycelia growth inhibition. Isolates Pf4 (79.8%), Pf13 (72.6%), Pf14 (70.3%), Pf19 (73%), and Pf20 (76.4%) all showed above 70% mycelia growth inhibition.

3.3 Effect of antagonists under greenhouse experiment

In the greenhouse experiment, native bacterial isolates applied to the soil were found to be beneficial in reducing the occurrence of wilt. In comparison to the control (24%), the bacterial antagonist presented in Table 2 and Figure 2 had the lowest incidence of wilt (4%) after 30 DAS in Pf4. However, when compared to the control (48%), isolate Pf14 had the lowest wilt incidence at 60 DAS (14.6%). After 90 DAS, the control had 80% of the wilted plants, whereas isolate Pf18 had the lowest incidence of wilt (30.6%), followed by Pf13 (33.4%), Pf14-20 (34%), Pf4 (34.6%), and Pf19 (39.4%), in that order. The strain Pf18 achieved the highest level of illness control (41.75%).

3.4 Effect of antagonists in the field experiment

The maximum shoot-root length and weight of the six isolates (Table 3 and Figure 3) were both measured in isolate Pf14 at 48 and 19.1 cm and 18.5 and 8.03 cm, respectively. Dowling and O'Gara (1994) also cited *Pseudomonas* spp. as the cause of the increased root elongation. In addition, the bacterial isolates enhanced the percentage of seeds that germinated (98-95%) in comparison to the control (89.9%), with isolate Pf13 exhibiting the highest seed germination. *P. fluorescens* isolates had a favorable impact on growth indices, as demonstrated by Rudresh *et al.* (2005). In Pf18 (5.5%; 30 DAS), Pf13-14 (22.7%; 60 DAS), and Pf14 (30.5%; 90 DAS), there were lower incidences of wilt than in controls (21.1, 59.4%, and 81.6%; correspondingly; after 30, 60, and 90 DAS).

Figure 1: Screening isolates of *P. fluorescens* against *F. oxysporum*.

Figure 2: Effect of selected *P. fluorescens* isolates on Fusarium wilt of chickpea in greenhouse condition.

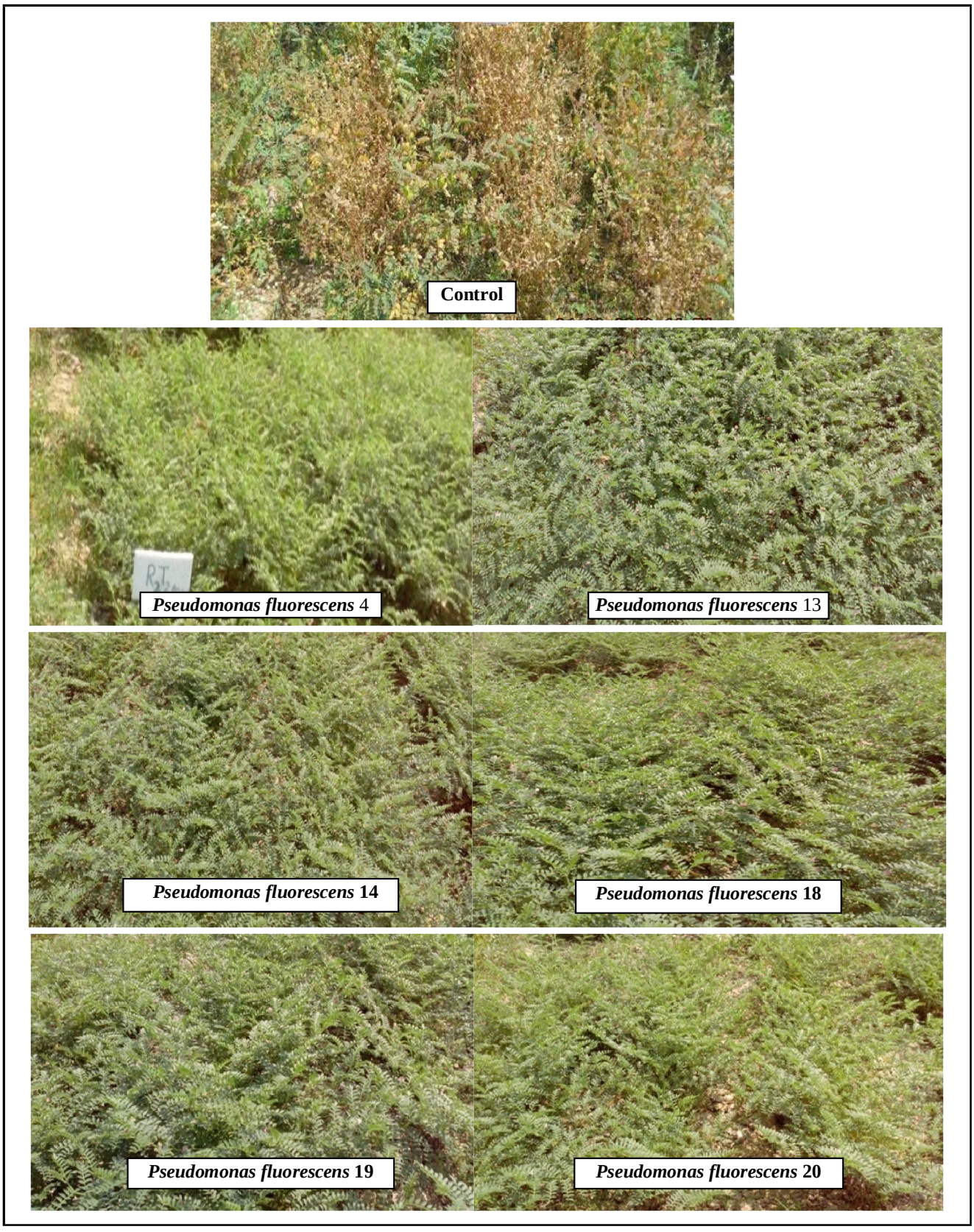

Figure 3: Effect of selected *P. fluorescens* isolates on Fusarium wilt of chickpea in the field trial.

Table1: ***In vitro*** **screening of** ***P. fluorescens*** **isolates against** ***F. oxysporum***

Isolates of *P. fluorescens*	Radial growth of *F. oxysporum* at 6th DAI	% Inhibition over control
Pf1	25.70	71.25
Pf2	25.26	71.74
Pf3	28.26	68.38
Pf4	9.43	89.45
Pf5	25.33	71.66
Pf6	21.06	76.44
Pf7	29.83	66.62
Pf8	28.96	67.60
Pf9	27.93	68.75
Pf10	27.40	69.35
Pf11	27.10	69.68
Pf12	29.46	67.04
Pf13	11.83	86.76
Pf14	21.86	75.54
Pf15	21.86	75.54
Pf16	28.03	68.64
Pf17	25.40	71.58
Pf18	8.90	90.04
Pf19	13.86	84.49
Pf20	14.40	83.89
Control	89.40	-
F-test	**S**	-
C.D. (*p* = 0.05)	**1.336**	-

Table 2: Effect of selected ***P. fluorescens*** **isolates on Fusarium wilt of chickpea in green house condition**

Treatments	% wilt incidence			% disease over control
	30(DAS)	60(DAS)	90(DAS)	
Control (inoculated)	24.00	48.00	80.00	-
Foc+Pf4	4.00	21.40	34.60	36.75
Foc+Pf13	16.00	26.00	33.40	38.25
Foc+Pf14	10.00	14.60	34.00	37.50
Foc+Pf18	6.00	28.60	30.60	41.75
Foc+Pf19	18.00	30.60	39.40	30.75
Foc+Pf20	8.00	18.60	34.00	37.50
SEd±	**0.30**	**0.16**	**0.17**	-
CD@ 5%	**0.60**	**0.28**	**0.35**	-

Table 3: Effect of selected *P. fluorescens* isolates on Fusarium wilt of chickpea in field trial

Treatments	% of seed germination	% wilt incidence (DAS)			Shoot length	Shoot weight	Root length	Root weight
		30	60	90	(cm)	(g)	(cm)	(g)
Control (inoculated)	89.90	21.20	59.40	81.60	31.03	8.70	12.53	3.57
Foc+Pf4	95.40	13.30	28.30	36.60	44.30	14.20	14.40	4.67
Foc+Pf13	98.00	8.80	22.70	31.10	45.30	15.20	15.30	5.37
Foc+Pf14	96.70	8.30	22.70	30.50	48.00	18.50	19.10	8.03
Foc+Pf18	97.10	5.50	23.30	31.60	45.00	15.50	14.80	6.50
Foc+Pf19	96.40	10.00	24.40	32.70	46.50	16.50	16.10	6.70
Foc+Pf20	97.40	6.60	27.70	36.10	44.20	14.27	13.30	5.20
SEd+	**3.69**	**0.28**	**1.57**	**1.53**	**0.21**	**0.18**	**0.19**	**0.69**
CD@ 5%	**8.07**	**0.85**	**4.72**	**4.59**	**0.64**	**0.53**	**0.58**	**1.77**

4. Discussion

The discovery was made by Krishnamurthy and Gnananamanikam (1998), who documented the antagonism of *Pseudomonas* spp. against several fungi both *in vivo* and *in vitro* situations. Goel *et al.* (2002) reported on a field investigation that identified *Pseudomonas* strains as promising biocontrol agents against *Rhizoctonia solani*, *Pythium* spp., and *F. ciceri* under culture conditions and field experiments. The extracellular release of an antifungal chemical by *P. fluorescent* was reported by Kumar *et al.* (2007). They also made a strong case for the importance of secondary metabolites like antibiotics and siderophores in the control of fungal infections.

The oil cakes of neem were shown to be the most efficient in inhibiting fungal development, according to Raj and Singh (1996) and Rahman *et al.* (2021). According to Govindachary (1992) and Patel *et al.* (2021) neem oilseed meals include several hazardous substances, while neem oil contains sulphur, resin, glycosides, and a number of acids, as well as desactylimbin, quercitin, sitosterol, and azardirachtion. For *F. solani*, Chakrabarti and Sen (1991) reported that neem oil cake had the highest growth inhibitory effects. In a greenhouse, Ha and Huang (2007) tested 10 organic compounds for preventing *F. oxysporum*-induced asparagus bean wilt.

Similar findings were made by Sakthivel and Gnanamanickam (1986), and *Pseudomonas* colonises on the root systems through seed bacterization and exhibits antagonistic behaviour toward *F. oxysporum*, *Rhizoctonia bataticola*, and *Pythium* spp. The same conclusion was reached by Anjaiah *et al.* (1998) and Vidhyasekaran and Muthamilan (1995).

Mane and Mahendra Pal (1998) and Kaur *et al.* (2007) and Selvarajan and Jeyarajan (1996) and others have all reported *Pseudomonas* ability to inhibit the chickpea root pathogen., The prevalence of various soilborne pathogens, including *F. oxysporum*, was significantly decreased by *P. fluorescens* isolates (Kaur *et al.*, 2003; Sandhu, 2001; Husain*et al.*, 2021).

Other researchers with similar findings (Izhar *et al.*, 1995; Siddiqui and Ehteshamul-Haque, 2001) showed significant disease suppression by *P. fluorescens* (23% root infection), which was also supported by Pandey and Chubey (2003) and Maurya *et al.* (2021) and Yadav *et al.* (2021). *Pseudomonas* was successfully used by Sharma *et al.* (1999) to reduce stem rot and root rot. In an *in vivo* investigation, Patel *et al.* (2011) found that Pseudomonas strains reduced disease incidence by 33 to 44% against *R.bataticola* and by 16 to 32% against *Sclerotinia sclerotiorum*.

Similarly, isolated *Pseudomonas* species from the chickpea rhizosphere were successful in controlling Fusarium wilt of chickpeas under controlled conditions (Hervas *et al.*, 1998; Landa *et al.*, 2001). According to Landa *et al.* (2004), *P. fluorescens* isolate RG 26 was the most effective treatment for controlling Fusarium wilt, delaying the development of the disease, and boosting seed output.

All five *Pseudomonas* strains affected Foc in a similar way, causing morphological alterations such as cytoplasmic granulation and condensation, cytoplasmic fragmentation, and hyphal deformation. The strains generated several substances that aided in the growth of plants, including cellulase, hydrogen cyanide, indole acetic acid, ammonia, siderophores, lipase, and solubilized phosphate. Additionally, they were able to dramatically boost chickpea development and decrease wilt illness (Khalifa *et al.*, 2022 and Kaur *et al.*, 2007; Rathore *et al.*, 2020).

P. fluorescens has been shown by Goel *et al.* (2002) to be a possible biocontrol agent for *R. solani*, *Pythium*, and *Fusarium oxysporum*. According to Anamika and Simon (2011), *P. fluorescens* treatment of the soil and seeds dramatically decreased the *F. ciceri* of chickpea and also increased shoot length, weight, root length, weight, and the number of grain pods per plant when compared to control (without treated plots).

5. Conclusion

Antagonistic effects of *P. fluorescens* against fungal *F. oxysporum* diseases was studied. Many strains of *P. fluorescens* have shown their potential towards suppressing the disease severity in chickpeas caused due to *F. oxysporum*. The present study aimed to evaluate operative strains isolated from chickpea fields. Out of twenty isolates of the native *P. fluorescens*, six isolates have the potential to the suppression of growth of *F. oxysporum*. These native isolates can be further utilized in the biological management of soil-borne pathogens in agricultural crop legumes.

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Conflict of interest

The authors declare no conflict of interest relevant to this article.

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