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IN VITRO EFFICACY OF PGPR *PSEUDOMONAS FLUORESCENS* AGAINST *FUSARIUM* WILT OF TOMATO CAUSED BY *FUSARIUM OXYSPORUM F.SP. LYCOPERSICI*

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ABSTRACT

Tomato (Solanum lycopersicum L.) is one of the most important, commercial and widely grown vegetable crop in the world. Tomato plays a critical role in nutritional food requirements, income and employment opportunities for the people. However, its production is threatened by the Fusarium wilt caused by *Fusarium oxysporum* f.sp. lycopersici and production losses between 30%to40%. In the present investigation an attempt has been made to study the *in vitro* efficacy of *Pseudomonas fluorescens* against *Fusarium oxysporum* f.sp. lycopersici. The antagonistic effect of *Pseudomonas fluorescens* were observed by the Dual culture technique and Agarwell method under the *in vitro* conditions.Among the ten isolates of *Pseudomonas fluorescens*, isolate Pf5 found to show the maximum percent inhibition over control (58.75%) and least mycelial growth (37.12mm) in dual culture technique against *Fusarium oxysporum* f.sp. lycopersici. In Agar well method isolate Pf5 proved out the maximum inhibition zone (17.47mm)against *Fusarium oxysporum*f.sp. lycopersici and percent inhibition over control (80.97%) at 30% concentration level.

Keywords: Tomato, Fusarium wilt, *Fusarium oxysporum* f.sp. lycopersici, *Pseudomonas fluorescens*, Dual culture technique, Agar well method.

INTRODUCTION

Tomato (*Solanum lycopersicum* L.) is one of the most cultivated and popular vegetable crop across the world (Pastor *et al.,* 2012). It belongs to the *Solanaceae* family and it is the most important vegetable after Potato (Gondal *et al.,* 2012). It is used as a fresh vegetable and may even be processed and canned as a paste, juice, sauce, powder or as an entire (Barone and Frusciante 2007). Tomato grows well in a relatively cool and dry climate, it is well adapted to all climatic zones around the globe. Tomato is used for consumption due to its high nutritive values, antioxidant and curative properties and it contains Vitamin A, Vitamin C and Vitamin E with 95.3% of Water, 0.07% Calcium and Niacin which have great importance in metabolic activities of humans (Sahu *et al.,* 2013). Tomatoes are excellent source of various micronutrients and antioxidants. It has high nutritional values which plays a crucial role in our daily home cooking (Singh *et al.,* 2019). Ripe tomatoes have high levels of Carotenoids, of which carotenes make up between 90 and 95% (Guil-Guerrero *et al.,* 2009). 'Lycopene' is a natural antioxidant produced only by the tomato that works effectively against the growth of the cancerous cells (Bhovomik *et al.,*2012). Tomato consumption has been associated with decreased risk of heart diseases, head and neck cancers and might be strongly protective against neurodegenerative diseases.

Tomato plants are susceptible to various diseases caused by different agents such as Bacteria, Viruses, Nematode ,Fungi and Abiotic factors (Sahu *et al.*, 2013). Among the fungal diseases, *Fusarium* wilt is caused by *Fusarium oxysporum* f.sp. *lycopersici* and it causes economic loss of tomato production in world wide. *F. oxysporum*f. sp. *lycopersici* is a soil borne pathogen, persists in soil for about 8-10 years in the form of chlamydospores as resting structure (Prachisingh *et al.,* 2019).The fungus *F. oxysporum*f. sp. *Lycopersici* is exerting pressure on production losses between 30 to 40% and may even raise upto80%if so, climatic conditions favour the growth of the fungus (Nirmaladevi 2016).

The symptoms of Fusarios is begin with a foliar chlorosis during a region of the plant and because the disease is established, the yellowing is observed within the majority of the plant, causing the wilt and later the death of the plant, without producing fruit or the fruit production is scarce (Baez-Valdez *et al.,* 2010).The earliest symptoms appear with in 48 h after the entry of the pathogens. In the infected plants the leaves becomes yellow followed by dropping of leaves which occurs may be on one side of the plant or on both the sides of shoot (Mui-Yun 2003b).

The PGPR having ideal potential to compact various pathogen, has been used in different forms of application. PGPR playing a vital role and capable of colonizing the plants root system and improve the growth and yield. Plant growth promoting rhizobacteria with biocontrol traits will be thought-about as another to the high doses of pesticides applied on crops to discourage the pathogens and cut back the sickness severity (Mahendra Prasad *et al.,* 2019). Mechanisms employed by PGPR square measure concerned in biocontrol like direct antagonism via production of Antibiotics, Siderophores, HCN, Hydrolytic enzymes or indirect mechanisms within which the biocontrol organisms act as a probiotic by competitory with the infectious agent for a distinct segment (Lugtenberg

Table 1: Isolation and cultural characteristics of various isolates of *Fusarium oxysporum*f.sp. lycopersici(Fol) from major tomato growing areas of Krishnagiri district

Sl. No.	Isolates	Locality	Cultural characteristics	Mycelial growth (mm)
1.	Fol_1	Hamumantheertham	Moderate Aerial mycelium cottony white to pink colour mycelium	86.76bc
2.	Fol_2	Irumathur	Aerial with white mycelium	87.56ab
3.	Fol_3	Uthangarai	Profuse fluffy cottony growth with white to pink mycelium	90.00a
4.	Fol_4	Thippampatti	Moderate aerial mycelium with white to pink mycelium	89.23b
5.	Fol_5	Puthoor	Moderate fluffy cottony growth with white mycelium	79.87f
6.	Fol_6	Arasur	Moderate aerial mycelium with slightly pink mycelium	74.35g
7.	Fol_7	Kollanaikanoor	Moderate fluffy cottony growth, white to pale pink colour mycelium	87.20c
8.	Fol_8	Mittapalli	Thin flat with slight pink colour mycelium	85.26cd
9.	Fol_9	Kodamandapatti	Fluffy white to pink colour mycelium	83.12e
10.	Fol_{10}	Mathur	Whitish fluffy growth, slightly pink colour	86.47d

* Mean of three replications
* In a column, means followed by a common letter are not significantly differ at 5% level by Duncan's multiple range test (DMRT)

Table 2: Morphological and biochemical characterization of native PGPR *Pseudomonas fluorescens*

Sl. No.	Locality	Colony Morphology	Isolates	Gram Staining	Starch hydrolysis	Nitrate reduction	KOH test	Fluorescent pigment
1.	Pochampalli	Smooth, Round, Shiny, Whitish	Pf1	-ve	-ve	-ve	-ve	+ ve
2.	Irumathur	Smooth, Round, Mucoid, Light green	Pf2	-ve	-ve	-ve	-ve	+ ve
3.	Uthangarai	Smooth, Round, shiny, off white	Pf3	-ve	-ve	-ve	-ve	+ ve
4.	Punganai	Smooth, Irregular, Mucoid, Light green	Pf4	-ve	-ve	-ve	-ve	+ ve
5.	Puthoor	Smooth, Round, Yellowish green	Pf5	-ve	-ve	-ve	-ve	+ ve
6.	Arasur	Smooth, Irregular, Shiny, Light green	Pf6	-ve	-ve	-ve	-ve	+ ve
7.	Kollanaikanoor	Smooth, Round, Shiny, Whitish	Pf7	-ve	-ve	-ve	-ve	+ ve
8.	Mittapalli	Smooth, Round, Shiny, Dull white	Pf8	-ve	-ve	-ve	-ve	+ ve
9.	Kodamandapatti	Smooth, Round, Bluish green	Pf9	-ve	-ve	-ve	-ve	+ ve
10.	Kallavi	Smooth, Irregular, Shiny, Whitish	Pf10	-ve	-ve	-ve	-ve	+ ve

* Mean of three replications
* In a column, means followed by a common letter are not significantly differ at 5% level by Duncan's multiple range test (DMRT)

Table 3: *In vitro* efficacy of *Pseudomonas fluorescens* against *Fusarium oxysporumf.sp. lycopersici*(Fol3)(Dual culture)

Sl. No.	Isolates	Mycelial growth (mm)	Percent inhibition over control (%)
1.	Pf1	52.46	41.71d
2.	Pf2	64.23	28.63 g
3.	Pf3	69.36	22.93i
4.	Pf4	72.65	19.27 h
5.	Pf5	37.12	58.75 a
6.	Pf6	42.87	52.36 b
7.	Pf7	56.78	36.91 e
8.	Pf8	47.58	47.13 c
9.	Pf9	45.56	49.37bc
10.	Pf10	60.87	32.36 f
11.	Control	90.00	-

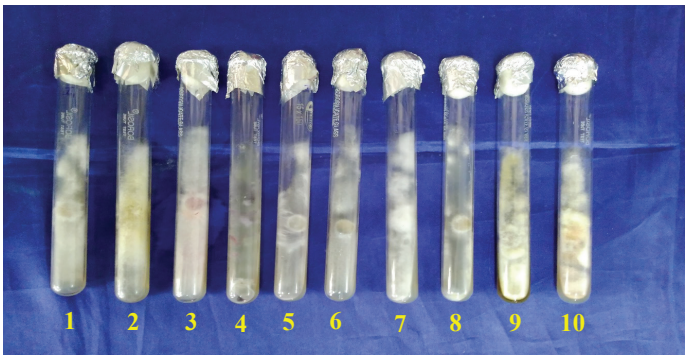
* Mean of three replications
* In a column, means followed by a common letter are not significantly differ at 5%level by Duncan's multiple range test (DMRT)

Table 4: Efficacy of *Pseudomonas fluorescens* against *Fusarium oxysporum*f.sp. lycopersici(Fol3) (Agar well method)

Sl. No.	Isolates	10%		20%		30%	
		Inhibition zone (mm)	Percent inhibition over control	Inhibition zone (mm)	Percent inhibition over control	Inhibition zone (mm)	Percent inhibition over control
1.	Pf1	11.21c	87.54	13.65bcd	84.83	16.64 cd	81.51
2.	Pf2	8.95ef	90.05	12.83ef	85.74	15.76fgh	82.48
3.	Pf3	8.45fg	90.61	12.59fg	86.01	15.59gh	82.67
4.	Pf4	7.87g	91.25	12.23g	86.41	15.24 h	83.06
5.	Pf5	12.95a	85.61	14.86 a	83.48	17.47a	80.58
6.	Pf6	12.43ab	86.18	14.49ab	83.90	17.12ab	80.97
7.	Pf7	10.25 d	88.61	13.19cde	85.34	16.21 de	81.98
8.	Pf8	11.86bc	86.82	13.94bc	84.51	16.81bcd	81.32
9.	Pf9	12.21b	86.43	14.13 b	84.30	16.97abc	81.14
10.	Pf10	9.37e	89.58	12.96def	85.60	15.97def	82.25
11.	Control	90.00	-	90.00	-	90.00	-

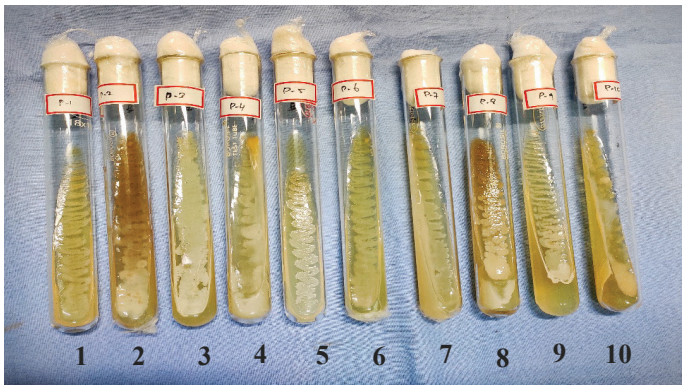
* Mean of three replications
* In a column, means followed by a common letter are not significantly differ at 5% level by Duncan's multiple range test (DMRT)

Plate 1: Different isolates of *Fusariumoxysporumf.*sp. *lycopersici*



1. Fol_1 2. Fol_2 3. Fol_3 4. Fol_4 5. Fol_5
6. Fol_6 7. Fol_7 8. Fol_8 9. Fol_9 10. Fol_{10}

Plate 2: Different isolates of *Pseudomonas fluorescens*



1. Pf_1 2. Pf_2 3. Pf_3 4. Pf_4 5. Pf_5
6. Pf_6 7. Pf_7 8. Pf_8 9. Pf_9 10. Pf_{10}

Plate 3: Efficacy of *Pseudomonas fluorescens* against *Fusarium oxysporum* f.sp. lycopersici(Dual culture)

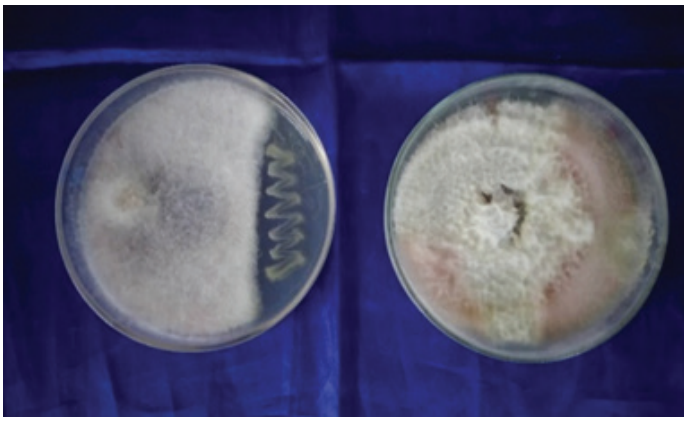
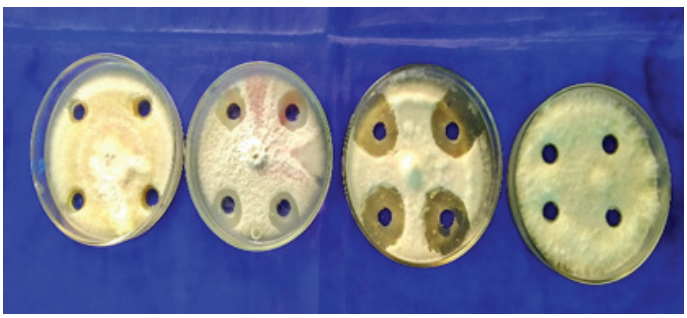


Plate 4: Efficacy of *Pseudomonas fluorescens* against *Fusarium oxysporum* f.sp. lycopersici (Agar well method)



et al., 2009).Isolates of *Pseudomonas* spp recovered from tomato rhizosphere were positive for HCN production which are able to control *Fusarium* wilt of tomato caused by *Fusarium* sp. (Lachisa *et al.,* 2015). *P. fluorescens* are the most exploited bacteria for biological control of soil-borne and foliar plant pathogens. In past three decades, numerous strains of *P. fluorescens* have been isolated from the rhizosphere soil and plant roots by several workers and their biocontrol activity against soil borne and foliar pathogens are reported (Vivekananthan 2004).

MATERIALS AND METHODS

Isolation and Identification of *Fusarium Oxysporum* F.sp. *Lycopersici*

Typical wilt symptom showing tomato plants were collected from different tomato growing areas of Krishnagiri district and used for isolation of pathogen. The infected root and stem portions were washed in tap water and the tissues showing vascular brown colour discolouration are cut into small pieces. They were then surface sterilized in 1% Sodium hypochlorite (NaOCl2) solution for 30 sec. To remove thetracesof Sodium hypochlorite solution the tissues were washed thrice with sterile distilled water and the pieces were transformed to the Petri plates containing sterilized potato dextrose agar (PDA) and incubated at room temperature(28 ± 2°C) for5-7days. The pure culture of pathogen is obtained by single hyphal tip method (Rangaswami 1972). Thepathogen *F. oxysporum*f.sp. *lycopersici* was identified with the help of descriptions given by Booth (1971).

Isolation of *Pseudomonas fluorescens*

For isolation of Rhizobacteria, the tactic followed by (Manasa *et al.,* 2017) was followed. during this procedure 10g of soil from every soil sample was taken in an exceedingly cone-shaped flask of 90ml water. The sample was agitated for quarter-hour on a vortex and serial dilutions of soil suspensions were ready. One millilitre of several dilutions was unfold on sterilized Petri plates of King's B medium. The petri plates were incubated at temperature (28 ± 2oC) for 24-72 h. The plates were examined daily up to three days for microorganism colonies.

Bio chemical test for *Pseudomonas fluorescens*

Gram staining

A loopfulof microorganism culture was transferred on a clean slide and a smear was created that was air dried and warmth mounted. The smear was flooded for 1min with ammonium ion oxylate bactericide. Excess stain was poured off and therefore the slide was washed in a very light steam of water. Lugol's iodine resolution was applied and allowed to stay for one min decolorized with alkyl radical alcohol. The smear was washed in a very light steam of water and counter stain with safranine for thirty seconds. The gram negative cells appeared red in color and gram positive cells appeared purple in color.

Starch Hydrolysis

Filter paper was dipped in a day old culture suspension and was placed on petri dishes containing starch agar medium and incubated for two days. The plates were flooded with one per cent iodine solution. A colourless halo around the growth and blue colour in the rest of the plates showed utilization of starch by the microorganism.

Nitrate Reduction

Inoculate the nitrate broths with microorganism suspension. Incubate the tubes at the best temperature 30^0c or 37^0C for twenty-four hours. when incubation explore for N_2 gas initial before adding reagents. Add 6-8 drops of nitrate chemical agent A and add the 6-8 drops of nitrate chemical agent B. Observe for the reaction (colour development) with a moment or less. If no color develops add metallic element powder. Observe for a minimum of three minutes for a red color to develop when addition of metallic element.

Koh Test

Apply one drop of three KOH on a microscopic slide. Use a loop to transfer a generous quantity of microorganism (Cultivated for 24-48h) to the drop of KOH. Stir fastidiously. the answer of gram negative microorganism are going to be viscous and type a mucoid string with in thirty sec.

Fluorescent Pigment

The test tube containing sterilized King's B medium were inoculated with the isolate of *Pseudomonas* spp. and incubated for five days and observed. Development of yellowish green fluorescent pigment observed under UV light indicated positive results.

Dualculture(Dennisandwebster1971)

A nine mm culture disc obtained from the periphery of the seven days old culture of *F. oxysporum*f.sp. *lycopersici* was inoculated at 75mm approximately away from the edge of the Petri dish containing 15ml of sterilized and solidified PDA medium. The bacterial antagonist *P. fluorescens* was streaked gently made onto the medium using two days old culture just opposite to the pathogenic culture at equidistance. The zone of inhibition (mm) and the mycelial growth of *F. oxysporum*f.sp.*lycopersici* were recorded. The effective antagonists were selected based on the inhibition to the growth of the pathogen. The per cent inhibition of mycelia growth was calculated according to Vincent(1927).

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

Where, I = Percent inhibition over control

C = Radial growth (mm) in Control

T = Radial growth (mm) in Treatment

Preparation of culture filtrate of *Pseudomonas fluorescens*

The effective *P. fluorescens* isolate were inoculated into Erlenmeyer flasks containing 50 ml of sterile King's B broth and kept on a rotary shaker at 100 rpm for 48 h. Then the cultures were filtered through bacteriological filter under vacuum and the filtrates thus obtained were used for the studies.

Agar well method (Schaad *et al.*, 2001)

Agar well diffusion method is also known as hole plate diffusion method (Brandt *et al.,*1993). This method was followed to determine the antagonistic activities of antibacterial metabolites present in *P. fluorescens* culture filtrate. Inoculated culture of 7 days old *P. fluorescens* was incubated in a shaker at 150 rpm for 2 days. Ensuing 10 ml of culture was centrifuged at 10,000 rpm for 20 minutes. The supernatant was filtered by using Whatmann No.1 filter paper and the spore suspension was collected.

The Petri plates amended with 15 ml solidified PDA medium was inoculated in the centre with 9 days old culture of *F. oxysporum*f.sp. *lycopersici*. Then, wells were cut equidistantly with the aid of the sterile cork borer. Different filtrate concentrations (10, 20 and 30µl) were suspended into four wells separated at a 2cm distance away from centrally placed *F. oxysporum*f.sp. *lycopersici*disc. Control plate without filtrate was maintained and incubated at (28 ± 2°C) for seven days to evaluate the maximum percent of inhibition. The inhibition zone formed around each well was measured and calculated.

RESULTS AND DISCUSSION

Isolation and Cultural Characteristics of Various Isolates of *Fusarium Oxysporum* F. Sp. *Lycopersici* (Fol) From Major Tomato Growing Areas of Krishnagiri District

The isolates of *F.oxysporum*f.sp. *lycopersici* showed variation with respect to colony characters. The Colour of the isolates varied from white to pale pink and pinkish. Most of the isolates produced fluffy to moderately fluffy cottony aerial mycelium (Table 1).Among the isolates of *F.oxysporum*f.sp. *lycopersici*the maximum mycelia growth (90.00mm) was recorded by the isolates Fol_3 isolated from Uthangari. The minimum mycelialgrowth (74.35mm) was recorded by the isolate Fol_6 isolated from Arasur (Table 1).

Rajendran *et al.,* (2018) reported that the pathogen produced different colony colors *viz*., Light pink, Pink, Dark pink, Creamy white, pale white with pink and the mycelial growth pattern showed two different pattern namely adherent smooth and fluffy growths.

Similarly, Amutha *et al.,* (2017) reported that such groupings have beendone with respect to *F. oxysporum*f. sp. *lycopersici*.In past studies, Nath *et al.,* (2017) mentioned that the colony colors *Fusarium oxysporum*f. sp. *lycopersici*were purplish white, Whitish orange, Creamy white, cottony white. Similar such groupings have been done with respect to *F.oxysporum*f.sp. *cubense* by Sanjeevkumar(2008); *F. oxysporum*f.sp. *lycopersici* by Sivakumar (2018). These early reports corroborate with the present findings.

Morphological And Biochemical Characterization Of Native Pgpr *Pseudomonas fluorescens*

A total ten isolates of *P. fluorescens* were able to be isolated from tomato rhizosphere in different localities of Krishnagiri District. The isolates of *P. fluorescens* produced smooth, Irregular to round in nature and it will be produced the different colours of colonies like viz., Dull white , Off white, white , Yellowish green , Light green , Bluish green. The results of the Gram Staining, Starch hydrolysis, Nitrate reduction, KOH test, Fluorescent pigment performed for the identification of the effective native isolates of *P. fluorescens* showed that all the isolates produced similar results with regard to Gram staining (negative), Starch hydrolysis (negative), Nitrate reduction (negative), KOH test (negative), Fluorescent pigment (positive). The identified isolates were designated as Pf_1 to Pf_{10}(Table 2).

Similarly Sundaramoorthy and Balabaskar (2013) mentioned that the different isolates of *Pseudomonas* produced different colony colors like White, Light yellow, Cream, Brownish green. The different species of *P. fluorescens* were characterized based on morphological, physiological and biochemical tests and they showed variation in colony colour, colony shape, margin, elevations and colony surface (Beeresh and Shripad 2018).

According to Wang *et al.,*(2015) reported that the different Morphology and biochemical characters *P.protegens* and *P.chlororaphis*. Similar observations made by Ketut Widnyana (2017) conducted the bio chemical test for different isolates of *P. alcaligenes*. In recent study made by Faeza *et al.,* (2019) characterization and biochemical tests for *P. fluorescens* RP13.

***In vitro* efficacy of *Pseudomonas Fluorescens* Against *Fusarium Oxysporum* F.sp. *Lycopersici* (Fol_3) (Dual Culture)**

In general all the native isolates of *P. fluorescens* tested significantly inhibited the mycelial growth of *F. oxysporum*f.sp. *lycopersici* (Table 3). However, among the isolates, the isolate Pf_5showed the maximum inhibition and significantly inhibited the growth of *F. oxysporum*f.sp. *lycopersici*(37.12mm), which was 58.75per cent reduction on the growth of the pathogen when compared to control. This was followed by the isolates Pf_6 and Pf_9 in the decreasing order of merit, which inhibited the growth of *F. oxysporum*f.sp. *lycopersici* by 52.36and 49.37per cent over control. The least growth inhibition of the pathogen (19.27%) was exhibited by the isolate Pf_4.

However the isolate Pf_5 of *P. fluorescens* significantly inhibited the *F. oxysporum*f.sp. *lycopersici*(Fol_3). The least growth inhibition of the pathogen was exhibited by the isolate Pf_4. In recent study Shanmuga Priya *et al.,* (2019) reported that the *in vitro* antagonism of ten isolates of *Pseudomonas* sp. against the mycelial growth of *Fusarium* sp. and indicated that the isolate *Ps* Ap was most effective in inhibiting the mycelial growth of virulent isolate of *Fusarium* spp (FI3) to the maximum of 57.77 percent.

Shahzaman*et al.,* (2016) reported, the bio efficacy of thirty isolates of *P. fluorescens* against the *Fusarium* sp. The antagonist Pf3 was found most effective with inhibition percentage of 93.33 per cent. Similar observations made by Vethavalli *et al.,* (2012)the efficacy of *in vitro* antagonism of three isolates (PV2, BVE1, and SM1), were the maximum inhibition percentage was observed in PV2 isolates revealed 77.22 per cent inhibition of growth of *F. oxysporum*f.sp. *lycopersici* over control.

In past studies Hannane *et al.,* (2016) concluded that the general inhibition is due to the combined effects of several extracellular and volatile compounds. According to Romanenko and Alimov (2000) mentioned where as*P. fluorescens*inhibits the development of the mycelium much more with volatile compounds.

Efficacy of *Pseudomonas Fluorescens* Against *Fusarium Oxysporum* F.sp. *Lycopersici* (Fol_3) (Agar Well Method)

The efficacies of culture filtrate of *P.fluorescens* against *F.oxysporum*f.sp. *lycopersici*by agar well method were depicted in the table4. The result showed that the culture filtrates of the *P. fluorescens*isolate Pf_5 recorded the maximum inhibition of *F.oxysporum*f.sp. *lycopersici*12.95mm, 14.86mm and 17.47mm with the percent inhibition of 85.61%, 83.48% and 80.58 % at 10%, 20% and 30% respectively. The minimum inhibition of *F.oxysporum*f.sp. *lycopersici* was noticed in the Pf_4 isolate with an inhibition of 7.87mm, 12.23mm and 15.24mm at 10%, 20% and 30% respectively.

In recent study Harshita *et al.,* (2019) revealed, the inhibition zone around the *P. fluorescens* against *F. oxysporum*f.sp.*lycopersici* with different concentrations (50 ppm,100 ppm, 200 ppm). Similar observations made by Vethavalli *et al.,* (2012) who reported that antagonist bacteria PV2 recorded the zone of inhibition (5.5 mm) at 10 µl concentration. Similarly, Zape *et al.,* (2014) reported that the *Pseudomonas* sp. culture filtrate containing wells to inhibit the *Fusariumsolani* mycelial growth due to production of antifungal compounds like Zwittermicin, 2, 4 – diacetyl-phloroglucinol etc.

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