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IN VITRO ANTAGONISM OF *PSEUDOMONAS* CF. *MONTEILII 9* AGAINST *FUSARIUM OXYSPORUM*, A CAUSATIVE AGENT OF FUSARIUM WILT DISEASE OF GROUNDNUT

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ABSTRACT:

Fusarium Wilt disease of groundnut is caused by Fusarium oxysporum Schlechtend. Emend Snyder & Hans., which leads to significant yield losses of crop. Control of fungal phytopathogens by using naturally occurring antagonistic microorganisms has been the focus of intense research throughout the world. This approach is popularly known as biological control of plant pathogens. Biological control is a bio-based, eco-friendly strategy which offers a practical and economical alternative for the management of plant pathogens with a potential to emerge as an alternative to chemical control. Pseudomonas spp .is one the candidate of the major fungal biocontrol agents found in the soil and the rhizosphere of various crop systems. 40 isolates of Pseudomonas were isolated from rhizosphere soil samples collected from various healthy ground nut - growing fields and were screened for in vitro antagonistic activity against F. oxysporum through dual culture technique. One isolate with promising antifungal activity against F. oxysporum was further identified through 16S rRNA sequencing as Pseudomonas cf. monteilii 9. Pseudomonas cf. monteilii 9 showed highest antagonism against F. oxysporum mycelia with the percentage inhibition up to 60.75%. The antifungal activities of Pseudomonas cf. monteilii 9 against F. oxysporum involved the secretion of volatile, non - volatile diffusible bioactive compounds, and siderophore.

KEYWORDS: *Fusarium* Wilt, Groundnut, *Fusarium oxysporum*, *Pseudomonas cf. monteilii 9*.

INTRODUCTION

Groundnut (*Arachis hypogaea* L.) crop is considered as most important crop in the World and is most important source of edible oil. Similarly, groundnut is ranked as 3rd most important source of vegetable protein in the world. Groundnut crop is prone to attack by many pathogens and to a much larger extent than many other crops. More than 100 pathogens, including viruses, have been reported to affect groundnut but only a few are economically important in India such as Leaf-spots (Tikka), Early leaf-spot (*Cercospora arachidicola*), Late leaf-spot (*C. Personatum*), Rust (*P. arachidis*), and aflatoxin contamination (*Aspergillus flavus* and *A. parasiticus*). The other diseases such as Collar rot (*A. niger*), Fusarium wilt (*Fusarium oxysporum*), Stem-rot (*S. rolfsii*), Root-rot (*M. phaseolina*), Bud necrosis (tomato spotted wilt virus), Clump and peanut (groundnut) mottle disease are localized[1].

Fusarium oxysporum is one of the most common soil fungi in cultivated soil all over the world. It includes a large diversity of strains, all saprophytic and most parasitic[2]. The wilt inducing strains of *F. oxysporum* cause a serious damage to many economically important agricultural crops[3]. *Fusarium* wilt pathogens are very host specific. Based on their host plant species and plant cultivars, there are more than 53 forms, 117 formae specials and 29 varieties[4]. *Fusarium* wilt is one of the common disease of groundnut, caused by the fungus *Fusarium oxysporum* lead to significant yield losses. The pathogen infects the roots and colonizes the vascular tissue, leading to wilting and finally death of the plant[5]. The management of *Fusarium*

wilt is mainly through chemical soil fumigation by methyl bromide. However, their use is costly as well as environmentally undesirable[6]. So, the use of soil fumigants has been banned because of their harmful effects on human health and environment[7].

The cost effective, environmental friendly newly emerging strategy of managing plant phytopathogen is the exploitation of biocontrol agents. Bacteria have been explored as biocontrol agents for plant diseases[8] and as plant growth promoters and inducers of disease resistance[9]. The rhizobacteriaidentified as potential biocontrol strains and plant growth promoting rhizobacteria often belong to the following genera (i) *Bacillus* spp.[10] (ii) *Pseudomonas spp.*[11] and (iii) *Streptomyces SP*[12].

The use of antagonistic bacteria has been reported as a powerful strategy to suppress soil-borne pathogens due to their ability to antagonize the pathogen by multiple modes and to effectively colonize the rhizosphere. The widely recognized mechanisms of biocontrol action include competition for an ecological niche or substrate, as well as the production of inhibitory compounds and hydrolytic enzymes that are often active against a broad spectrum of fungal pathogens. Many microorganisms are known to produce multiple antibiotics which can suppress one or more pathogens[13]. For instance, *Bacillus subtilis* produces several ribosomal and non-ribosomal peptides that act as antibiotics such as iturins, surfactins and zwittermycin[14] and it secretes also hydrolytic enzymes, i.e. protease, glucanase[15], chitinase[16], lipase[17] and amylase[18]. In many instances, *Pseudomonas sp.* and *Bacillus sp.* have been applied as biocontrol agents to suppress plant-pathogenic organisms[19]. *Bacillus sp.* are gaining recognition as safe biocontrol agents in a variety of crops, specifically as seed protectants and antifungal agents[14]. Moreover, they are spore-formers, which impart a natural formulation advantage over other microorganisms[20].

The present work aimed to search for effective antagonistic rhizobacteria for controlling *Fusarium oxysporum*, the causative agent of Fusarium wilt disease of groundnut. Keeping in view, the importance of rhizospheric bacteria in sustainable agricultural development by controlling the phytopathogen, the present study aims at (i) Isolation of rhizospheric bacteria from different rhizospheric source, (ii) Screening of rhizospheric isolates isolated from rhizospheric soil for in vitro antagonism against *Fusarium oxysporum*(iii) To identify the rhizospheric isolate based on 16SrRNA sequencing and (iv) Characterization of *in vitro* biocontrol mechanism of Rhizospheric isolates.

MATERIALS AND METHODS

Fusarium Wilt Phytopathogen:

The *Fusarium oxysporum,* causing Fusarium wilt in groundnut, was procured from the earlier work carried at Department of Microbiology, S. G. B. Mahavidyalaya, Purna.

Isolation of *Pseudomonas sp.* from Rhizospheric soil:

Rhizospheric soil from healthy groundnut plants was collected in poly-ethylene bags and brought to the research laboratory. 1 gm of soil sample was inoculated into 100 ml nutrient broth and kept for incubation at room temperature for 24 h.

For isolation of *Pseudomonas* sp., 1ml of this nutrient broth was transferred to selective enrichment media such as Cetrimide broth and kept for incubation at room temperature for 24 h. From enriched Cetrimide broth, a loopful of culture was streaked on Cetrimide agar[21], and the plates were incubated at room temperature till colonies were observed (24 – 48 h). The isolated colonies developed were then purified on nutrient agar slants and used for screening purpose against the phytopathogen for biocontrol ability.

Screening for Potential Biocontrol agents:

All the isolates of *Pseudomonas sp.* were screened for potential antagonistic activity against *Fusarium oxysporium* on King's B agar plate[22] through dual culture technique[23]. A 5-mm disc was cut from an actively growing *Fusarium oxysporium* culture on Nutrient agar plate and placed on the surface of fresh King's B agar medium at the one side of the Petri plates. A loopful of actively growing *Pseudomonas* sp. (each) was placed opposite to the fungal disc. The control plates were prepared by inoculating King's B agar plate only with phytopathogen. Each experiment was carried out in triplicates. Plates were incubated at room temperature for 7 days. Degree of antagonism was determined by measuring the radial growth of pathogen with bacterial culture and control. Percent inhibition was calculated by the following equation[24].

Percent Inhibition = Diameter of control – Diameter of Test / Diameter of Control

Where,

Control = Colony diameter of Pathogen alone in mm

Test = Colony diameter Pathogen+Antagonist in mm

Identification of Antagonistic *Pseudomonas sp*.:

An effective antagonistic *Pseudomonas sp*. obtained through screening against *Fusarium oxysporum*, was identified with the help of 16s rRNA sequencing commercially carried at Agharkar Research Institute (ARI) Pune, Maharashtra.

Characterization of Antagonistic Mechanism:

To characterize the antagonistic mechanism shown against *Fusarium oxysporum* during screening, the efficient *Pseudomonas* sp. were tested to produce Volatile metabolite, Non – volatile diffusible metabolite and Siderophore.

Detection of Volatile Metabolites production:

The volatile antifungal metabolites production by the antagonistic bacteria was detected by using method where two half plates (sterile) was used[25]. The two plates were poured with sterile molten and cooled Kings B Agar and allowed to solidify. Now the *Pseudomonas* culture was spot inoculated on one of the half plate in centre and on the other half of the plate, a disc of four-day old pure culture of *Fusarium oxysporum* was placed. Both half plates were placed face to face preventing any physical contact between the pathogen and the bacterial culture.The two plates were sealed together with sticky tape to minimize loss of volatiles produced. Control plates were prepared in a similar manner but the bottom plate contained King's B agar without a bacterial culture. The plates were incubated at room temperature for 6 days and the growth of the pathogen was measured and compared to control plates developed in the absence of the antagonist. Experiment was run in triplicate. Results expressed in terms of inhibition (%) of fungal growth in the presence and absence of bacterial isolate and percent inhibition was calculated by using formula[26].

$$\textbf{Percent Inhibition} = \frac{(\textbf{Control} - \textbf{Test})}{\textbf{Control}} \textbf{100}$$

Where,

C - Radial growth of fungus in control plates (mm).

T - Radial growth of fungus on the plate inoculated with Antagonist (mm).

Detection of Non – Volatile Diffusible Metabolite:

To check Non-volatile diffusible metabolite production with slight modification instead of PDA plates King's B plates were used[26]. The plates covered with a cellulose nitrate membrane, were inoculated in the center with *Pseudomonas* suspension. After incubation for 48 h at room temperature, the membrane with grown bacterial culture was removed, and the plate was inoculated in the center (below the paper) with a 5-mm disk of *Fusarium oxysporum* and plates were re-incubated at room temperature for 7 days and the growth of the pathogen was measured. Control were run with un-inoculated King's B plates containing plates on the cellulose nitrate membrane (replacing the bacterial suspension by sterile distilled water), and further incubated with pathogen *Fusarium oxysporium*. Experiment was run in triplicates. Results were expressed as % inhibition of fungal pathogens in the presence and absence of antagonistic bacterial isolate[26].

Analysis of Siderophore Production:

The *Pseudomonas* sp. inoculum was prepared in King's B medium and incubated at 28 ^{o}C on rotary shaking incubator (120 rpm) for 18-20 h.

Siderophore production was carried out using modified Succinate medium[27]. 0.1 ml of inoculum was separately inoculated in 250 ml Erlenmeyer flask containing Succinate medium and incubated on rotary

shaker incubator for 48 h at 28°C. After incubation, supernatant was harvested by centrifuging the culture at 10,000 rpm in cooling centrifuge at 4°C for 10 min.

Siderophore Detection:

Siderophore production was detected using Universal Chrome Azurol S Assay (CAS)[28]. Here equal volume of supernatant and CAS reagent was mixed in a clean test tube and observed for color change as compare to reference.

EXPERIMENTAL RESULTS:

Isolation of *Pseudomonas* sp. from Rhizospheric soil:

In present work, 40 rhizospheric *Pseudomonas* sp. was isolated from healthy groundnut rhizospheric soil and were tentatively named as *Pseudomonas* isolate 1 to *Pseudomonas* isolate 40 to avoid confusion.

Screening for Potential Biocontrol agents:

All the *Pseudomonas* isolates displayed some antagonistic activity against *Fusarium oxysporum* by dual culture technique. In our studies all isolates of *Pseudomonas* inhibited growth of *F. oxysporum* to various extents. A maximum inhibition percent of 60.75 was measured for *Pseudomonas* isolate 29 and minimum inhibition percent of 10.98 was found for isolate 20 (Table 1 and Photo Plate 1) and this isolate was used for further study.

Identification of efficient antagonistic *Pseudomonas* sp.:

The most efficient antagonistic *Pseudomonas* isolate 29 spotted during screening against *Fusarium oxysporum* by dual culture technique was identified by 16S rRNA sequencing as *Pseudomonas cf. monteilii 9* with accession number AF181576 of GenBank.

Characterization of Antagonistic Mechanism:

The bacterial agents exert a protective effect on plant through antagonism towards phytopathogenic fungi. Two major mechanisms have been proposed to explain the suppressive and antagonistic effects of *Pseudomonads* i.e. either the phytopathogen is inhibited by competition for iron, as availability of Fe^{+++} in soil is low (10^{-18} M) nor secondly *Pseudomonas* inhibit the pathogens by producing secondary metabolites with antagonistic activity e.g. Phenazine, Pyrrolnitrin, 2,4-diacetylphloroglucinol and cyanides.

Though, the disease suppression is multifunctional attributes, hence for understanding the mechanism of action shown by the *Pseudomonas* isolate under study, variety of experiments were carried out during this research work. To elucidate the antagonistic mechanism, *Pseudomonas cf. monteilii 9* were tested for production of Volatile metabolite, Non- Volatile diffusible antibiotic and Siderophore. From the results, it was evident that *Pseudomonas cf. monteilii 9* produced variety of secondary metabolites namely non-volatile diffusible antibiotic, volatile metabolites and Siderophore (Table 2).

Detection of Volatile Metabolites production:

The volatile antifungal metabolites production by the antagonistic bacteria was detected after 6 days incubation *Pseudomonas cf. monteilii 9* produced volatile metabolites to inhibit *Fusarium oxysporium*, 73.23 % (Photo Plate 2 and Table 3).

Detection of Non – Volatile Diffusible Metabolite:

To elucidate Non-Volatile Diffusible Metabolite production results were recorded after 7 days of incubation where *Pseudomonas cf. monteilii 9* secrete non-volatile diffusible metabolites and causes 100% inhibition of *Fusarium oxysporum* on King's B medium (Photo Plate 3).

Siderophore Detection:

Siderophore production was detected using Universal Chrome Azurol S Assay (CAS)[28]. *Pseudomonas cf. monteilii 9* gave instant color change of CAS reagent from blue to classical golden orange when tested qualitatively by Universal CAS assay (Photo Plate 4).

DISCUSSION:

Pseudomonas sp. has also been reported as being antagonistic to several plant pathogenic organisms such as, *Macrophomina phaseolina*[31], *Fusarium oxysporum*[5], *F. oxysporum* f. sp. *lycopersici*[32] and *F. oxysporum* f. sp. *lycopersici*[29]. *Pseudomonas fluorescens* CHA0 applied for control of *Fusarium oxysporum* f. sp. *lycopersici* where they found 50 % inhibition in dual culture method[33]. Fluorescent Pseudomonads have been exploited to control root diseases caused by fungal pathogens: isolates of *P. fluorescens, P. putida, P. aeruginosa* and *P. aureofaciens* suppress soil-borne pathogens through rhizosphere competition[35], antibiosis[36] and iron chelation by production of siderophores[37]. *P. fluorescens* WCS365 and *P. putida PCL1760* suppressed tomato foot and root rot in stonewool[38]. This literature survey supported that *Pseudomonas* sp. could be efficiently exploited to control the phytopathogen, *Fusarium oxysporum*.

While, comparing these above results with our results, which are far better than these results, where *Pseudomonas* isolate 29 inhibited *Fusarium oxysporum* up to 60.75 % in *vitro* studies. later on *Pseudomonas* isolate 29 was identified by 16S rRNA sequencing as *Pseudomonas cf. monteilii* 9.

To illustrate the biocontrol mechanism, *Pseudomonas cf. monteilii* 9 was tested for production of secondary metabolites such as Volatile metabolites, Non – Volatile Diffusible Metabolites and Siderophore Production.

Volatile secondary metabolites such as ammonia[39] and HCN[40] were produced by many rhizosphere strains including *Pseudomonas* strains and have been implicated as important metabolites in biocontrol[41]. *P. fluorescens CHA0* an aerobic, root-colonizing biocontrol bacterium that protects several plants from root diseases caused by soil-borne fungi especially by the production of HCN[40]. HCN production by strain CHA0 contributes to the suppression of a disease caused by *Thielaviopsis basicola.* The productions of ammonia by *Enterobacter cloacae* strain have contributed in the control of Phytopathogen, *Pythium ultimum* and *Rhizoctonia solani in vitro*[39]. Volatile compounds such as ethylene and ammonia gas produced by rhizospheric bacteria exert their effect by inhibiting the germination of fungal spores[42].

Literature survey reveals that our results were more promising when compared with the results earlier[43] reported for control of *Phytophthora capsici*. Our results with *Pseudomonas* appears better than the above-mentioned result because there was only 2-23 % and 36-70 % inhibition recorded where as our result was 73.23% inhibition for the tested pathogens. These results were promising for biocontrol of phytopathogenic fungi of groundnut.

Similarly, the biocontrol abilities of *Pseudomonas* sp. mainly depend on aggressive root colonization, induction of systemic resistance in the plant, and production of antifungal antibiotics[44]. The production of one or more antibiotics is the mechanism most commonly associated with the ability of plant growth-promoting bacteria to act as antagonistic agents against phytopathogens[45]. The basis of antibiosis activity of biocontrol based on, secretion of molecules that kill or reduce the growth of the target pathogen[46]. Antibiotics encompass a heterogeneous group of organic, low-molecular-weight compounds that are deleterious to the growth or metabolic activities of other microorganisms[47]. Antagonistic micro-organisms, *Pseudomonas* sp. produces a wide range of different antimicrobial and antifungal secondary diffusible metabolites, such as 2,4-diacetylphloroglucinol (DAPG), pyrrolnitrin (PRN), pyoluteorin (PLT), phenazines (PHZs), and cyclic lipopeptides[48] and hydrogen cyanide, which is volatile[49]. These antimicrobial compounds may act on plant pathogenic fungi by inducing fungistasis, inhibition of spore germination, lysis of fungal mycelia or by exerting fungicidal effects.

Pyrrolnitrin, the antibiotic produced by the *P. fluorescens* BL915 strain, can prevent the damage of *Rhizoctonia solani* during damping-off of cotton plants[50]. The 2,4-diacetylphloroglucinol (DAPG) produced by Pseudomonads, an effective and extensively studied antibiotic, causes membrane damage to *Pythium* sp. and is particularly inhibitory to zoospores of this oomycete[51]. Phenazine, also produced by Pseudomonads, possesses redox activity and can suppress pathogens of plants such as *F. oxysporum* and *Gaeumannomyces graminis*[52]. The *P. chlororaphis* PCL1391 strain, isolated from roots of tomato plants, synthesizes phenazine-1-carboxamide, which can release soluble iron from insoluble ferric oxides at neutral pH, raising the possibility that phenazines might contribute to iron mobilization in soils[49].

DAPG is believed to be a major determinant in the biological control of plant diseases by *Pseudomonas* sp., such as take-all disease of wheat caused by *Gaeumannomyces graminis var. tritici* (Ggt) and black root rot disease of tobacco caused by *T. basicola*[53].

Our results were in accordance to the results documented earlier i.e. *Pseudomonas cf. monteilii 9* produced non-volatile diffusible antibiotic which inhibited *Fusarium oxysporium*. All these results supported that *Pseudomonas* strains produced non – volatile diffusible metabolite which might include Phenazine-1-carboxylic acid (PCA), 2,4-diacetylphloroglucinol (2,4-DAPG), Pyoluteorin (PLT), Pyrrolnitrin (PRN) which needs to be confirmed further.

Establishment of the importance of siderophore production as a mechanism of biological control of *Erwinia carotovora* by several plant growths promoting *Pseudomonas fluorescens* strains A1, BK1, TL3B1 and B10[54]. A Pseudobactin siderophore produced by *P. putida* B10 strain was also able to suppress *Fusarium oxysporum* in soil deficient in iron, a condition that represses the production of iron chelator by microorganisms[54]. Recent studies have demonstrated the suppression of soil-borne fungal pathogens through the release of iron chelating Siderophore by fluorescent Pseudomonads, making it unavailable to other organisms[55]. Siderophore production confers competitive advantages to PGPR that can colonize roots and exclude other microorganisms from this ecological niche[49].

These results showed like other siderophore producing fluorescent *Pseudomonas* sp., *Pseudomonas cf. monteilii 9* produced Siderophore which played a vital role in antagonism of *Fusarium oxysporium in vitro.*

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Table 1: *In Vitro* screening for Antagonistic agent against *Fusarium oxysporum* by Dual Culture Technique

Isolates	Inhibition of *Fusarium oxysporum* (%)	Isolates	Inhibition of *Fusarium oxysporum* (%)
Pseud. Isolate 1	12.80	*Pseud.* Isolate 21	24.98
Pseud. Isolate 2	43.00	*Pseud.* Isolate 22	28.90
Pseud. Isolate 3	11.70	*Pseud.* Isolate 23	47.85
Pseud. Isolate 4	18.00	*Pseud.* Isolate 24	54.73
Pseud. Isolate 5	21.70	*Pseud.* Isolate 25	40.75
Pseud. Isolate 6	14.90	*Pseud.* Isolate 26	21.65
Pseud. Isolate 7	27.34	*Pseud.* Isolate 27	37.75
Pseud. Isolate 8	34.00	*Pseud.* Isolate 28	55.70
Pseud. Isolate 9	17.00	***Pseud.* Isolate 29**	**60.75**
Pseud. Isolate 10	17.00	*Pseud.* Isolate 30	38.95
Pseud. Isolate 11	32.76	*Pseud.* Isolate 31	47.00
Pseud. Isolate 12	33.78	*Pseud.* Isolate 32	36.98
Pseud. Isolate 13	12.78	*Pseud.* Isolate 33	53.27
Pseud. Isolate 14	19.42	*Pseud.* Isolate 34	46.03
Pseud. Isolate 15	21.80	*Pseud.* Isolate 35	41.05
Pseud. Isolate 16	34.87	*Pseud.* Isolate 36	39.27
Pseud. Isolate 17	37.89	*Pseud.* Isolate 37	38.04
Pseud. Isolate 18	40.00	*Pseud.* Isolate 38	36.75
Pseud. Isolate 19	53.75	*Pseud.* Isolate 39	37.21
***Pseud.* Isolate 20**	**10.98**	*Pseud.* Isolate 40	13.78

Table 2: Characterization of Antagonistic mechanism of *Pseudomonas cf. monteilii 9* against *Fusarium oxysporum*

Pseudomonas Isolate	Volatile Metabolites (VM)	Non – Volatile Diffusible Metabolite (NVDM)	Siderophore
Pseudomonas cf. monteilii 9	+++	+++	+++

+++= Efficient; ++= Satisfactory; += Average; - =Negative

Table 3: Volatile Metabolite Production by *Pseudomonas cf. monteilii 9* against *Fusarium oxysporum*

Pseudomonas isolate	Diameter of Control (mm)	Diameter of Test (mm)	Percent Inhibition
Pseudomonas cf. monteilii 9	85	10	73.23

Captions of figures

CONTROL

TEST

Photo Plate 1: Inhibition of *Fusarium oxysporum* by *Pseudomonas* isolate 29 during screening by dual culture technique.

CONTROL

TEST

Photo Plate 2: *In Vitro* production of Volatile metabolite by *Pseudomonas cf. monteilii 9* against *Fusarium oxysporum*

CONTROL

TEST

Photo Plate 3: *In Vitro* Non- Volatile Diffusible Metabolite production by *Pseudomonas cf. monteilii 9* against *Fusarium oxysporum*

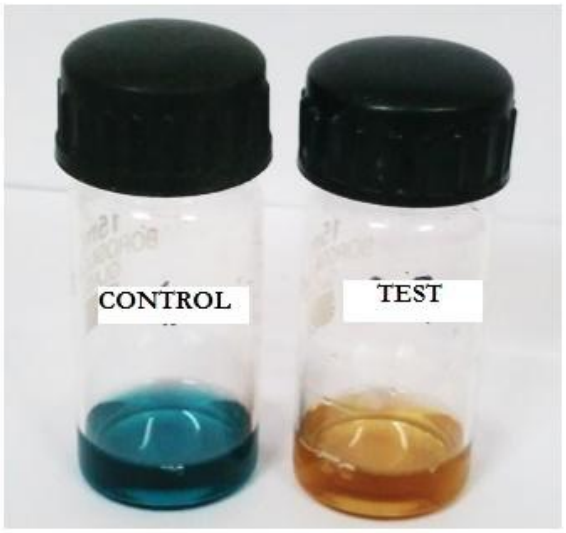

Photo Plate 4: *In Vitro* Siderophore Production by *Pseudomonas cf. monteilii 9*