Secondary Metabolites and Lytic Enzymes Produced by Fluorescent Pseudomonads Determine *in-vitro* Antagonistic Potential Against *Sclerotium rolfsii* in Groundnut (*Arachis hypogaea* L.)

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Stem rot caused by *Sclerotium rolfsii,* is a potential threat to many crop plants including groundnut (*Arachis hypogaea* L.) production world-wide. To understand the biochemical basis of *in vitro* antagonistic potential of Pseudomonads against *S. rolfsii*, eleven fluorescent Pseudomonads isolates (*SKPf1- SKPf11*) were biochemically evaluated. Molecular identifications through 16S sequencing revealed that the isolates were differing at their species level but all belonged to the large and heterogeneous group of fluorescent Pseudomonads. Based on the antagonistic potential, *SKPf5* was identified to be the superior among all which was capable of producing higher amount of antifungal metabolites and growth promoting compounds like such as siderophores, salicylic acid (SA) and indole acetic acid (IAA). Besides this, in *in vitro* study *SKPf5* also produced higher amount of lytic enzymes such as chitinases, β-1,3 glucanase and protease; and was better capable of inhibiting the activity of fungal polygalacturonase and cellulase as compared to other isolates. Association among different biochemical traits revealed that potentially better antagonistic Pseudomonads isolates were capable of producing higher amount of SA and IAA with better efficiency to degrade fungal cell wall through production of more chitinase and ß-1,3 glucanase. Hence these traits can be used for potential antagonistic fluorescent Pseudomonads identification and selection criteria against many fungal pathogens.

Key words: 16S rDNA, lytic enzymes, secondary metabolites, *in-vitro* antagonism.

Peanut (*Arachis hypogaea* L.), also known as groundnut, is an important food as well as an oilseed crop of the tropical and sub-tropical world. As a rich source of energy (564 kcal 100g⁻¹), it contains about 48–50% oil, 25–28% proteins and 20-26% carbohydrates [1]. India is one among the leading producers of groundnut worldwide. With an annual production of about 9.67 MT, India ranks second in the world after China in production [2]. Stem rot, caused by *Sclerotium rolfsii*, is a

potential threat to groundnut production in many warm, humid areas; especially it has emerged as one of the most potential disease in India. Due to aberrant rainfall pattern, shifting cultivation and unavailability of varieties showing absolute resistance against this disease, has sometimes led to a heavy economic loss to the growers.

Stem rot has wide host range, and is capable of producing several non-specific metabolites, oxalic acid and several cell wall degrading enzymes, hence it is logical to predict a low probability for success in finding useful levels of host resistance. This may be one of the reasons for the relatively low emphasis placed on breeding

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groundnuts for resistance to *S. rolfsii* [3]. At present no groundnut cultivars are known to have absolute resistant to *S. rolfsii*. Few groundnut genotypes such as GG 16, Dh 8, OG-52-1, CS 19 and CS 319 were reported to have lesser yield loss due stem rot disease [4, 5]. As with most soil-borne fungal pathogens, stem rot disease management also involves exclusionary practices, plant removal, soil removal or treatment, plant treatment, crop rotation, use of resistant varieties, or a combination of these practices. The specific management practices used depends upon the crop and cropping situation. For control of this disease, growers prefer chemical applications followed by use of biocontrol agents.

Management through chemical methods leads to ill effects like residual toxicity, environmental pollution and fungicide resistance. Although seed treatment with fungicides is recommended to minimize the infection at early stages, it does not give prolonged protection. Once established in the soil, it is difficult to eliminate this pathogen. Soil application of fungicides is difficult because of its broad host range as well as its worldwide distribution which precludes such strategy. Biological control i.e. application of beneficial microorganisms to soil, seeds or planting materials has been proposed as a sustainable and supplementary approach to control plant diseases [6]. In recent years this strategy has been proved to be a promising disease-management technology especially against soil-borne plant pathogens. The most widely studied microorganisms with antagonistic activity against plant pathogens and with beneficial effects on plant growth, belong to the bacterial genera *Bacillus*, *Pseudomonas, Rhizobium,* or the fungal genus *Trichoderma* [7, 8, 9]. For biocontrol of *S. rolfsii,* among the identified bacterial genera and species, most of them belong to the genera *Pseudomonas* and *Bacillus* [10]. Antagonistic *Pseudomonas* strains can restrict *in vitro* hyphal growth or reduce germination of sclerotia of *S. rolfsii* [11, 12, 13, 14]. The inherent antifungal activity of *Pseudomonas* strains indicates the possibility of using them as a biological control agent against *S. rolfsii*.

It is well known that all the isolates of biocontrol agents such as Pseudomonads are not equally antagonistic toward a particular species of pathogen. This emphasizes the need for identifying isolates specifically effective against *S. rolfsii*, for a planned regional deployment. Hence the present study was undertaken to isolate, identify an effective fluorescent pseudomonads isolate from the groundnut growing areas of *Saurasthra* region of Gujarat, potentially capable of inhibiting *S. rolfsii*. Further, the experiment aimed at identifying biochemical markers which can be used as a tool for quick and easy characterization and identification of suitable *Pseudomonas* isolates against this fungal pathogen.

MATERIAL AND METHODS

Isolation and maintenance of microbes

Fluorescent pseudomonads strains were isolated from soil samples collected from the groundnut rhizosphere grown in different areas of *Saurasthra* region, Gujarat, using King's B media [15, 16]. One gram of each soil sample was mixed by shaking for 2 hrs on a rotary shaker at 200 rpm in 10 mL of sterile water and the diluted extracts were distributed on King's B (KB) medium in petridishes. After incubation at 28 ^{0}C for 24 hrs, colonies that showed fluorescence under UV light were selected. The representative types of colonies were further purified on KB agar medium and pure isolates have been maintained in 50% glycerol at -20 ^{0}C. Arbitral names were assigned such as *SKPf 1* to *SKPf 11* and henceforth the names have been used in the text. Similarly, the pure culture of the fungus was isolated from *S. rolfsii* infected stem of groundnut plants [16]. The small pieces of the tissues were surface sterilized with 0.1 % mercuric chloride solution for 30 sec, washed and then plunged in potato dextrose agar (PDA) medium in petridishes. These petridishes were kept for incubation at 28 ± 2 ^{0}C in an incubator. A typical white mycelial growth was observed after 48 hours of incubation. This mycelial growth was further purified by "single spore isolation technique" and confirmed as *Sclerotium rolfsii*. Pure culture of the fungus has been maintained on PDA slant at 0-5 ^{0}C.

Molecular identification of fluorescent Pseudomonad isolates using 16s sequencing

The genomic DNA of bacteria was isolated from over-night grown bacterial culture [17]. One mL of the culture in Luria Broth (LB) medium was centrifuged at 10,000 g for 5 min. After removing the supernatant, the cells were washed with 400 µL STE Buûer (100 mM NaCl, 10 mM Tris/

HCl, 1 mM EDTA, pH 8.0) twice. Then the cells were centrifuged at 10,000g for 2 min. The pellets were resuspended in 200 µL TE buûer (10 mM Tris/HCl, 1 mM EDTA, pH 8.0). Then 100 µL Tris-saturated-phenol (pH 8.0) was added to these tubes, followed by a vortex-mixing for one minute. The samples were subsequently centrifuged at 13,000g for 5 min at 4 ^{0}C to separate the aqueous phase from the organic phase. About 150 µL of the upper aqueous phase was transferred to a clean 1.5 mL tube and 50 µL TE buûer was added to make 200 µL and mixed with 100 µL chloroform and centrifuged for 5 min at 13,000g at 4 ^{0}C. The process was repeated again to ensure the purification of nucleic acids. Then the upper aqueous phase was transferred to a clean 1.5 mL tube and RNAse treatment was given by adding 40 µL TE and 5 µL RNase (at 10 mg. mL^{-1}) and incubated at 37 ^{0}C for 10 min to digest RNA. Then 100µL chloroform was added to the tube, mixed well and centrifuged for 5 min at 13,000g at 4 ^{0}C. About 150 µL of the upper aqueous phase was transferred to a clean 1.5 mL tube which contained the puriûed bacterial DNA and was stored at -20 ^{0}C for further use. The 16S rRNA gene was amplified using the universal primer pair of 8F (5'-AGAGTTTGATCC TGGCTCAG-3') and 1492R (5'-GGTTACCTTGT TACGACTT-3')[18]. The expected size (~1500bp) of the amplified PCR products was resolved on 1 % (w/v) agarose gel, and later purified and sequenced directly using a Taq DyeDeoxy Terminator Cycle Sequencing kit (Applied Biosystems) at Food Testing Laboratory, Junagagadh Agricultural University, Junagadh. For reconfirmation, few samples were sequenced at SciGenome Facility, Cochin. The amplified 16S DNA of all selected isolates was sequenced and sequence similarity analysis was performed using BLAST (Blastn) search tool (http://www.ncbi.nlm.nih.gov). Based on the similarity (more than 95%) of the 16S sequences of the isolates to the already available sequences in NCBI database, Pseudomonad species were identified. The sequences were further checked for chimera sequences and submitted to the NCBI.

In-vitro per cent growth inhibition of *S. rolfsii*

The *in-vitro* antagonistic potential of the fluorescent Pseudomonad isolates against *S. rolfsii was* carried out using King's B media [18]. Each isolates in round shape and mycelial disc of 4

mm diameter from test fungus were placed in centre of the test medium in the same petridish approximately 4 cm away from each other. All the inoculated petridishes were incubated at 28 ± 2 ^{0}C and observed after six days for growth of antagonist bacteria and test fungus. Index of antagonism was determined by following the method [19] of as depicted below

$$\% \text{ Growth Inhibition} = \frac{C\text{-}T}{C} \times 100$$

Where,
C = colony diameter of pathogen in control, T = colony diameter of pathogen in inhibition

Cell wall degrading enzymes assay

Crude enzyme extract was prepared from the petridishes containing fungal pathogen and isolates (T$_1$ to T$_{11}$ where T$_n$ = *SKPf$_n$* X *S. rolfsii*) as well as the test fungus alone as a control (C) at 6 days after inoculation (DAI). For that, 25 mL of 100 mM phosphate buffer (pH 5.5) containing 50 mM sodium chloride was added to each petridish and transferred the whole mycelia mat along with the bacterial growth to a conical flask. For enzyme induction, one per cent of each of carboxymethyl cellulose (CMC), sodium polypectate, chitin or casein was added into culture medium of conical flask and the pH was adjusted to 5.5 [20], and then the cultures were shaken well in an orbital shaker at 120 rpm at 30 ^{0}C for about 6 hrs. After that, it was transferred to 30 mL tubes and centrifuged at 15,000 rpm for 10 min. Supernatant was collected and volume was made up to 100mL with the 100mM phosphate buffer, pH 5.5 containing 50 mM sodium chloride, and stored at -20 ^{0}C until it was used for protein estimation and assay of enzymes activities. Estimation of Cellulase and Polygalacturonase activity were measured spectrophotometrically [21, 22]. Estimation of Chitinase and β-1, 3 glucanase activities was measured by the net formation of sugar N-acetylglucosamine [23, 24], which was measured by DMAB method [25]. The values depicted in the text are normalised by deducting the respective C value referring to the *S. rolfsii* alone. Specific activity of the cell wall degrading enzymes and pathogenesis related enzymes were expressed as U mg^{-1} protein. However, unit activity was defined as the amount of enzyme necessary to produce one mM of corresponding reducing sugar per min per mL of culture supernatants. Respective controls were also

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performed using boiled enzymes for getting the exact sample values. For proteases activity, the standard method was followed [26] and specific activity of the enzyme was expressed as U mg^{-1} protein. One unit of protease activity was defined as the amount of protein necessary to produce mg free amino acids per min per mL of culture supernatant. Estimation of released free amino acids was made by the ninhydrin method [27]. The method of Folin-Lowry [28] was used to determine the protein content in the enzyme extracts. The standard curve for protein content was calculated by using Bovine serum albumin as standard, keeping the range of protein from 20-200 µg µl^{-1}.

Estimation of secondary metabolites

The Pseudomonads isolates were grown in KB medium at $28 \pm 2\ ^0$C for 72 hrs on a rotary shaker. The supernatant fraction was collected by centrifugation at 10,000 rpm for 10 min and used for estimation of the secondary metabolites production by the Pseudomonads isolates. Spectrophotometric estimation of siderophores [19] was carried out as outlined by Reddy *et al.* (2008) and the quantity of siderophore synthesized was expressed as µ mol benzoic acid mL^{-1} of King's B culture broth. The standard curve was prepared using dihydroxy-benzoic acid. Similarly, spectrophotometric based estimation of IAA-like substances (IAA) [29] and Salicylic acid (SA) [19] were carried out and the quantity in the culture filtrate was expressed as ng mL^{-1} and mg mL^{-1} respectively. Three independent replications were maintained for the estimation of each parameter.

Statistical analysis

Analysis of variance (ANOVA) was performed using DSAASTAT (Dipartimento di Scienze Agrarie ed Ambientali STATistics, ver. 1.101) and the significance of differences among means was carried out using Duncan's multiple range tests (DMRT) at $P < 0.05$ [30]. The correlation between different traits was studied using PAST v1.89 software [31].

RESULTS AND DISCUSSIONS

Isolation and molecular identification of fluorescent Pseudomonads

Eleven strains of fluorescent pseudomonads forming unique colony were isolated from groundnut rhizosphere based on their capability to give prominent fluorescence under UV light. The 16S sequencing followed by their sequence similarity search analysis revealed that all the fluorescent Pseudomonad isolates used in experiment were belonging to the genera *Pseudomonas* (Table 1) but none of them are *P. fluorescens*. Among the 11 isolates, five (*SKPf 1, SKPf 4, SKPf 8, SKPf 10,* and *SKPf 11)* were *P. putida*, three (*SKPf 2, SKPf 3,* and *SKPf 6)* were *P. monteilii*, two (*SKPf 7* and *SKPf 9)* were *P. plecoglossicida* and one (*SKPf 5)* was belonging to *P. aeruginosa*. The fluorescent Pseudomonads is a large and heterogeneous group comprises, most notably, *P. aeruginosa*, *P. putida*, *P. fluorescens* and *P. syringae* etc. [32]. All the members of this group give fluorescence under UV light as they are capable of producing the fluorescent pigment Pvd (also known as pseudobactin). The taxa *Pseudomonas* include both biocontrol agents and strains without any obvious biocontrol potential, regardless of whether only true *P. ûuorescens* or also related ûuorescent Pseudomonads are considered [33]. Hence, the selected fluorescent Pseudomonad isolates were further tested *in vitro,* for their antagonistic potential against the stem rot causing pathogen of groundnut.

In-vitro* antagonistic potential of fluroscent Pseudomonad isolates against *S. rolfsii

The *in-vitro* growth inhibition of *S. rolfsii* by the isolates such as *SKPf 1* to *SKPf 11* was observed at six days after inoculation (6 DAI) which revealed that all the isolates were not capable of inhibiting the fungus. The per cent growth inhibition was significantly different among the potential and non-potential antagonist (Table 1). Based on the *in-vitro* antagonism study, the maximum inhibition was brought by *SKPf 5* against *S. rolfsii*, followed by *SKPf 2, SKPf 4,* and *SKPf 11* which were at par. Based on BLASTN results of partial 16S sequence, the *SKPf 5* was identified as *P. aeruginosa,* where as both *SKPf 4* and *SKPf 11* were found to be *P. putida*. Previously, it was reported that some strains of *P. aeruginosa* also inhibit the *S. rolfsii* of groundnut [20], which supports the finding and suggests the potential of some *P. aeruginosa* isolates as biocontrol agent in groundnut.

It was observed that different species of ûuorescent Pseudomonads have different antagonistic activity. The result also revealed the

fact that the bacteria belonging to the same species may have different ability to inhibit the growth of pathogens, for instance, although both *SKPf 1* and *SKPf 4* were identified as *P. putida,* only the later showed fungal growth inhibition activity. Similarly, *SKPf 10* did not show any *in vitro* inhibition, where as *SKPf 11* showed better inhibition against the fungus, although both isolates belong to *P. putida.* In an attempt to develop effective biocontrol system for management of stem rot disease in groundnut, few *Pseudomonas* spp. isolated from rhizospheric soil, were evaluated for their antagonistic activity against *S. rolfsii*. The most potential soil bacterium showing highest antagonistic activity against *S. rolfsii* was identified as, *P. monteilii* [13]. However, in the present experiment, none of the isolated *P. monteilii*, did show any antagonistic activity against the same test fungus. Biological control using antagonistic Pseudomonads has been proven to be a potential

Table 1. Fluorescent Pseudomonad isolates with their NCBI accession number and size of submitted sequences

Isolate Name	Species of Fluorescent Pseudomonad Isolates	NCBI Accession No.	*In vitro* growth inhibition Potential (%) ± SE
SKPf 1	*Pseudomonas putida*	KR422298	1.90 ± 1.4 c
SKPf 2	*Pseudomonas monteilii*	KR422299	22.2 ± 2.0 b
SKPf 3	*Pseudomonas monteilii*	KP859615	2.30 ± 1.2 c
SKPf 4	*Pseudomonas putida*	KR492887	16.6 ± 3.5 b
SKPf 5	*Pseudomonas aeruginosa*	KP859614	33.7 ± 2.4 a
SKPf 6	*Pseudomonas monteilii*	KP859616	1.92 ± 1.4 c
SKPf 7	*Pseudomonas plecoglossicida*	KP859617	3.50 ± 1.4 c
SKPf 8	*Pseudomonas putida*	KR422300	1.10 ± 0.7 c
SKPf 9	*Pseudomonas plecoglossicida*	KR492888	1.90 ± 1.4 c
SKPf 10	*Pseudomonas putida*	KR422301	2.30 ± 1.8 c
SKPf 11	*Pseudomonas putida*	KR492889	17.2 ± 4.5 b

Values in the column followed by the same letters are not significantly different according to DMRT at P < 0.05 and are mean of three replications, SE refers to Standard error

Table 2. Production PR proteins and inhibition of fungal lytic enzymes by fluorescent Pseudomonad isolates

Treatments	Pathogenesis related enzymes (U mg^{-1} protein)			% Inhibition of cell wall degrading enzymes	
	Chitinase	β-1,3 Glucanase	Protease	Cellulase	Poly-galacturonase
SKPf 1 X *S. rolfsii* (T_1)	0.95	65.40	25.07	37.87	48.29
SKPf 2 X *S. rolfsii* (T_2)	1.63	108.75	9.19	30.51	39.70
SKPf 3 X *S. rolfsii* (T_3)	0.58	37.71	16.81	57.41	40.43
SKPf 4 X *S. rolfsii* (T_4)	1.84	5.88	11.44	49.17	58.64
SKPf 5 X *S. rolfsii* (T_5)	4.94	200.35	30.89	62.26	62.63
SKPf 6 X *S. rolfsii* (T_6)	2.74	99.54	1.31	37.22	50.75
SKPf 7 X *S. rolfsii* (T_7)	1.15	83.62	2.71	36.38	45.27
SKPf 8 X *S. rolfsii* (T_8)	0.67	32.53	19.46	42.93	50.48
SKPf 9 X *S. rolfsii* (T_9)	0.96	68.66	24.33	33.90	46.04
SKPf 10 X *S. rolfsii* (T_{10})	1.32	46.65	1.89	25.29	52.72
SKPf 11 X *S. rolfsii* (T_{11})	1.99	16.08	18.81	23.83	56.14
S.Em.±	0.14	6.79	1.23	2.39	2.87
LSD (p<0.05)	0.42	19.8	3.56	6.96	5.91
CV (%)	11.99	0.42	10.98	11.34	7.64

Values in the column are mean of three replications, S.Em.± refers to Standard error of mean, LSD (p<0.05) is Least Square Difference at P d" 0.05 and CV (%) refers to Coefficient of variation in percentage

Table 3. Association study between different biochemical traits

	% Growth Inhibition	SA	IAA	Siderophores.	Chitinases	Glucanases	Proteases	PG	Cellulases
% Growth Inhibition	0.000								
Salicylic Acid (SA)	0.769**	0.000							
Indole Acetic Acid (IAA)	0.199	0.435	0.000						
Siderophores	0.208	0.320	0.599*	0.000					
Chitinases	0.770**	0.900**	0.616*	0.426	0.000				
Glucanase.	0.533	0.857**	0.271	0.071	0.746**	0.000			
Proteases	0.344	0.462	0.197	0.058	0.228	0.238	0.000		
Polygalacturonases(PGs)	0.492	0.522	0.787**	0.493	0.684	0.143	0.268	0.000	
Cellulase	0.305	0.547	-0.089	-0.075	0.375	0.354	0.443	0.222	0.000

management tool for reducing the severity of groundnut stem rot [11]. Many researchers proved that selected *Pseudomonas* strains can restrict *in vitro* hyphal growth or reduce germination of sclerotia of *S. rolfsii* [11, 12, 20, 34, 35].

Production of lytic enzymes

Many fluorescent Pseudomonads are reported to produce lytic enzymes and the main roles of these enzymes for defence reaction are evident from the fact that they degrade the cell wall because chitin and ß-1,3-glucan are also major structural component of the cell walls of many phytopathogenic fungi. Among the successful biocontrol agents, many *Trichoderma* species capable of producing these lytic enzymes kill sclerotia or mycelium of *S. rolfsii* through lysis [36]. Hence, it was hypothesized that the superior biocontrol isolate should produce higher amount of fungal cell wall degrading enzymes.

Chitinase activity

Chitinase produced by biocontrol agents like fluorescent Pseudomonads degrade the chitin, which is a major integral part of many fungal
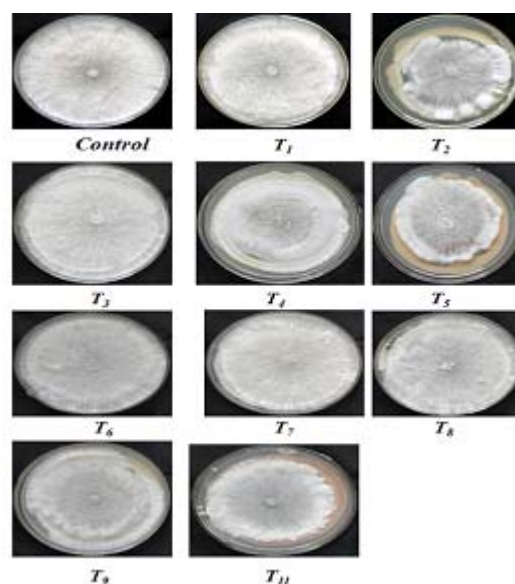

Fig. 1. *In-vitro* antagonism between isolates of fluorescent Pseudomonads and *S. rolfsii* at 6 DAI in King's B medium, where Control: *S. rolfsii* alone ; T_1: *SKPf 1* X *S. rolfsii* ; T_2: *SKPf 2* X *S. rolfsii* ; T_3: *SKPf 3* X *S. rolfsii* ; T_4: *SKPf 4* X *S. rolfsii* ; T_5: *SKPf 5* X *S. rolfsii* ; T_6: *SKPf 6* X *S. rolfsii* ; T_7: *SKPf 7* X *S. rolfsii* ; T_8: *SKPf 8* X *S. rolfsii* ; T_9: *SKPf 9* X *S. rolfsii* ; T_{10}: *SKPf 10* X *S. rolfsii* and T_{11}: *SKPf 11* X *S. rolfsii*

pathogens. In the present study, all isolates produced the chitinase and there was a significant difference among the isolates for their capability to produce the enzyme against *S. rolfsii in vitro* (Table 2 and Fig 1). The mean of specific activity of chitinase was ranged between 0.58 U (*SKPf 3*) to 4.94 mg^{-1} protein (*SKPf 5*) which was in accordance with their *in vitro* growth inhibition ability against *S. rolfsii*. Although *SKPf 6* could
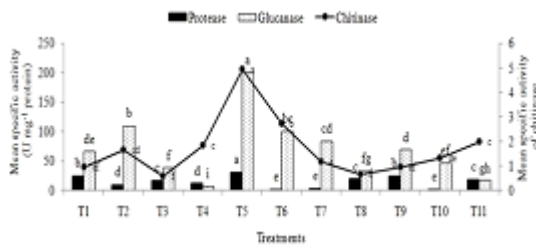
Fig. 2. *In-vitro* production of Protease, â-1, 3-glucanase and Chitinase by fluorescent Pseudomonads isolates against *S. rolfsii* (T$_1$ to T$_{11}$), the value of each column represents the mean of three independent replications and the treatments having same letter on respective column show no significant difference at p<0.05.
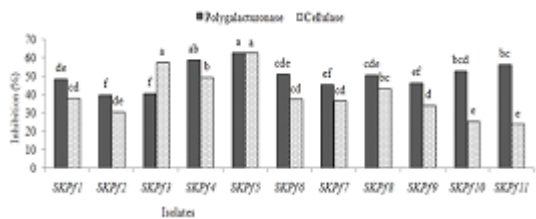
Fig. 3. *In-vitro* inhibition of fungal Polygalacturonase and Cellulase activity by fluorescent Pseudomonad isolates, the value of each column represents the mean of three independent replications and the isolates having same letter on respective column show no significant difference at p<0.05.
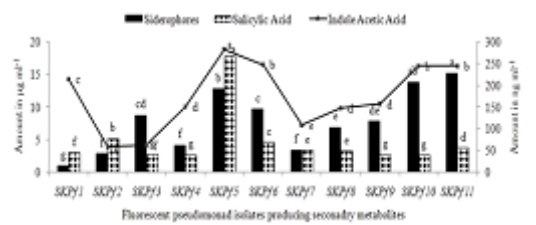
Fig. 4. *In-vitro* production of secondary metabolites by fluorescent Pseudomonads isolates, the value of each column represents the mean of three independent replications and the isolates having same letter on respective column show no significant difference at p<0.05.

not able to inhibit *S. rolfsii* growth, but appreciably more amount of chitinase (2.74 U mg^{-1} protein) was produced by the isolate than others. It was also observed that mostly, those isolates having no or little antagonistic behaviour were associated with less amount of chitinase production *in-vitro*. In an earlier study an isolate of *P. aeruginosa,* capable of producing higher extracellular chitinase was reported to be a better biocontrol agent against *S. rolfsii* [37]. Our finding were supported by the fact that potentially better strains produce higher amount of chitinase [38], indicating their vital role in inhibiting the *in-vitro* growth of the pathogen. It was also in agreement with some earlier findings [39] who reported a positive association between the antagonistic potential of *P. fuorescens* strains and their level of chitinase production.

β-1, 3 glucanase activity

The enzyme β-1,3-glucanase produced by the biocontrol agents like *P. fluorescens* have a capacity to hydrolyze the branched β-1,3-glucans found in the most fungal cell walls [40]. All the tested isolates produced significant amount of the enzyme against *S. rolfsii*, and the specific activity was in the range of 5.88 to 200.35 U mg^{-1} protein. The maximum specific activity of β-1,3-glucanase was reported by *SKPf 5* followed by *SKPf 2* (Table 2 and Fig 1). Although a signiûcant relationship between the antagonistic activity of *P. fluorescens* strains and their ability to produce β-1, 3-glucanase generally exist [41]; however we could not find such results. We observed potential isolates such as *SKPf 4* produced the less amount of β-1, 3-glucanase compared to the inefficient strains like *SKPf 6, SKPf 7* and *SKPf 9*. A negative or non-significant association between production of â-1,3-glucanase and *in-vitro* antagonistic potential of *Pseudomonas* isolates was also reported earlier [39]. Based on the finding of chitinase or β-1, 3-glucanase, it can be hypothesized that fluorescent pseudomonad isolates adapt differently to inhibit the growth of fungal pathogens by modulating their lytic enzymes production. Hence, selection of potential *in-vitro* antagonistic isolates of fluorescent Pseudomonads against fungal pathogens such as *S. rolfsii* should be avoided only considering their efficiency to produce chitinase or β-1, 3-glucanase.

Protease activity

Filamentous fungal cell wall contains lipids and proteins; hence it is most likely that the proteases produced by many antagonistic bacteria like fluorescent Pseudomonads might be acting on the cell wall proteins of fungal pathogens. It has also been hypothesised that the proteases might be degrading the cellulolytic enzymes produced by the pathogenic fungi, thereby reducing the fungal virulence [42]. In the present study, we too observed higher production of protease activity (30.89 U mg^{-1}protein) by the best antagonist isolate (*SKPf 5)* and the least activity was for *SKPf 6* and *SKPf 10* corresponding to the non-antagonist. However, few non potential isolates such as *SKPf 1* (25.07 U mg^{-1}protein) and *SKPf 9* (24.33 U mg^{-1}protein) (Table 2 and Fig 1) also produced significant amount of proteases. Hence it can be proposed that the *in vitro* antagonistic potential of Pseudomonads isolate are not solely depend upon their capacity to produce proteases.

Inhibition of fungal cell wall degrading enzymes

Plant fungal pathogens especially necrotrophic fungus degrade the structural polymers in plant host cell wall and colonize in the intercellular spaces facilitated by the production of cell wall degrading enzymes (CWDEs) such as cellulase, and polygalacturonase. However, potential biocontrol agents, such as bacteria like *Pseudomonas* spp. prevent the production of these CDWEs and thereby reduce the cell wall damage to the host tissues. In the present study, the inhibition of fungal CDWEs by all the isolates was observed (Table 2 and Figure 2). The per cent inhibition of cellulase activity of *S. rolfsii* was in the range of 23.83 % to 62.26% corresponding to the isolates *SKPf 11* and *SKPf 5* respectively. The inhibition of cellulase production by *SKPf 3* was at par with that of the best antagonist (*SKPf 5)*, however all other isolates inhibited cellulase activity of the fungus significantly less. Similarly, all the isolates showed the inhibition of polygalacturonase production of the test fungus *in vitro*. The maximum inhibition was recorded for *SKPf5* where was the least was for *SKPf 2*. Potentially better antagonist other than *SKPf 5* such as *SKPf 4* and *SKPf 11* significantly inhibited the production of polygalacturonase which was higher than other isolates. Cell- free culture ûltrates

of *P. aeruginosa* GSE 18 and GSE 19 reported to inhibit the *in vitro* activity of CWDEs produced by *S. rolfsii* up to a maximum of 55% and 50%, respectively [20], when measured 6 days after inoculation. Supporting thee earlier reports, it can be proposed that inhibitory activity of fluorescent Pseudomonad isolates on production of CWDEs activity of fungus plays an important role in *in-vitro* antagonism.

Production of secondary metabolites
Production of siderophores

Siderophores are fluorescent, yellow-green, water soluble, low molecular weight iron chelators secreted by many *P. fluorescens* isolates under iron-limiting conditions [43]. The *in vitro* secretion of siderophores by isolates of fluorescent Pseudomonads has been correlated with their corresponding antagonistic potential [29, 16]. In the present study, all *Pseudomonas* isolates reported to produce siderophores (Figure 3), the maximum production was recorded for *SKPf 11* (15.13 µg benzoic acid.mL^{-1}), where as lowest was for *SKPf 1* (0.99 µg benzoic acid.mL^{-1}). Although siderophores production by *SKPf 5* (12.75 µg benzoic acid mL^{-1}) was less than the above two, it was significantly higher than that of all other isolates. Although siderophore production is most often associated with fungal suppression by fluorescent *Pseudomonas* in the rhizosphere, but the result here revealed that it may not hold true for all isolates and all pathogens.

Production of Salicylic Acid

Under deprivation of iron, many Pseudomonads produce Salicylic acid (SA) which plays a vital role of SA in *in-vitro* antagonism [41, 16]. SA is a phenolic compound that affects a variety of biochemical and molecular events associated with induction of disease resistance through Induced Systemic Resistance (ISR). We observed that the SA production by the fluorescent Pseudomonad isolates was in the range of 2.61 µg mL^{-1} to 17.77 µg mL^{-1} (Figure 3). The capability of *SKPf 5* to produce higher SA compared to all other isolates possibly explains its superior antagonistic behaviour against the *S. rolfsii*.

Production of Indole acetic acid

Many isolates of fluorescent Pseudomonads possess intrinsic ability to produce Indole acetic acid (IAA) [29, 44, 45]. Isolates of *P. fluorescens* isolated from groundnut

rhizosphere reported to produce IAA and enhance the plant growth [46]. The present study found that all the isolates of fluorescent Pseudomonads produced IAA, but with variable quantity (Figure 3). The IAA production capacity of the tested isolates was in the range of 59.32 (*SKPf 2*) to 282.84 ng.mL^{-1} (*SKPf 5*). Although the production of IAA by *SKPf 6, SKPf 10,* and *SKPf* 11 were at par, and was significantly less than that of *SKPf 5,* the best antagonist among all the tested isolates. Many researchers also reported the capability of *P. aeruginosa* isolates to produce IAA [47]. The results depicted here did not some of the earlier findings [16] suggesting that the antagonistic behaviour of *Pseudomonads* depend upon their *in vitro* capability to produce IAA.

Association between different biochemical traits

Correlation study among all the observed traits (Table 3) revealed that *in-vitro* growth inhibition potential of a fluorescent isolate depends on its capability to produce SA acid and Chitinase. Positive association between SA production and fungal cell wall degrading enzymes suggests the SA as a marker to identify antagonistic Pseudomonads. A positive association was observed between IAA production and siderophore production, suggesting an antagonistic isolate of Pseudomonad could be equally potential for growth promotion in plants. Further there was a highly significant association between IAA production and capacity of an isolate to inhibit the production of polygalacturonase by the fungus, *S. rolfsii*. Hence, it can be hypothesized that a plant growth promoting bacteria works both by providing growth promoting hormone as well as protecting the plant cell wall maceration by inhibiting the production of fungal PG, which otherwise degrade the plant cell wall. A significant association between production of IAA and chitinase, further extends the hypothesis that growth promoting effect of some IAA producing Pseudomonads may be enhanced by their capability to produce chitinase, which degrades the degrades rhizospheric fungal pathogens. A significant association was noticed between production of chitinase and glucanases, both of which are associated with degrading the fungal cell wall.

CONCLUSION

Among the tested isolates of fluorescent Pseudomonads, the most potential antagonist i.e. *SKPf 5* was capable of producing higher amount of antifungal metabolites and growth promoting compounds like IAA. Although some inefficient antagonists also produced a significant amount of one and/or the other secondary metabolites, but except *SKPf 5*, all isolates are not equally good in producing siderophores, SA and IAA. The capacity of *SKPf 5* to produce those secondary metabolites constantly better than the average and the possible synergistic effects of all those metabolites on *in vitro* antagonism would have made the isolate better than the others. A similar logic can be put forth to justify the antagonistic potential of *SKPf 5* and its capacity to produce PR proteins and to inhibit the fungal cell wall degrading enzymes. In *in-virto* condition, *SKPf 5* produces maximum amount of pathogenesis related enzymes which logically made it the best antagonist against the *S. rolfsii* among all isolates. All though some isolates were individually good at producing one or the other enzymes to degrade the fungal cell wall, but not equally good as *SKPf 5,* for instance, growth inhibition potential is concerned. Similarly, *SKPf 5,* also is the best among the studied isolates for inhibiting the ability of *S. rolfsii* to produce cell wall degrading enzymes. Irrespective of the ability to inhibit the fungal growth *in vitro*, some isolates were individually also good at producing one or more of these enzymes. Hence from this study, it is deciphered that a potential biocontrol agent must be superior in production of PR proteins as well as should possess capability to inhibit the fungal produced cell wall degrading enzymes. Association among different biochemical traits revealed that potentially better antagonistic Pseudomonads isolates are capable of producing higher amount of SA and IAA with better efficiency to degrade fungal cell wall through production of more chitinase and glucanase, and hence the traits can be used as a marker for identification and selection.

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