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ORIGINAL ARTICLE

Biocontrol Potential of Siderophore Producing Heavy Metal Resistant *Alcaligenes* sp. and *Pseudomonas aeruginosa* RZS3 vis-à-vis Organophosphorus Fungicide

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Abstract In present study in vitro phytopathogen suppression activity of siderophoregenic preparations of Ni and Mn resistant *Alcaligenes* sp. STC1 and *Pseudomonas aeruginosa* RZS3 SH-94B isolated from soil were found superior over the chemical pesticide. Siderophore rich culture broth and siderophore rich supernatant exerted antifungal activity against *Aspergillus niger* NCIM 1025, *Aspergillus flavus* NCIM 650, *Fusarium oxysporum* NCIM 1281, *Alternaria alternata* ARI 715, *Cercospora arachichola*, *Metarhizium anisopliae* NCIM 1311 and *Pseudomonas solanacerum* NCIM 5103. Siderophore rich broth and supernatant exhibited potent antifungal activity vis-à-vis oraganophosphorus chemical fungicide; kitazine. The minimum fungicidal concentration required was 25 µl for *Aspergillus niger*, *Aspergillus flavus*, *Fusarium oxysporum*, *Cercospora arachichola*, *Metarhizium anisopliae*, *Pseudomonas solanacerum* and 75 µl for *A. alternata.*

Keywords *Alcaligenes* sp. · *Pseudomonas aeruginosa* RZS3 · Siderophores · Phytopathogen · Biocontrol · PGPR

Introduction

The growing cost of pesticides and consumer demand for pesticide-free food has led to a search for substitutes for these products. In this regards siderophore producing plant growth promoting rhizobacteria (PGPR) has been recognized as effective biocontrol agent against plant pathogens. Though the siderophores are specific ferric ion chelator, but they can also bind other metals. Thus, heavy metal contaminated soil can be largely influenced by siderophores.

Heavy metals are metals with a density above 5 g/cm^3 [1]. Presence of heavy metals even in traces is toxic and detrimental to both flora and fauna. PGPR capable of growing in presence of variety of heavy metals are seen as potent bioinoculants [2]. Bioabsorption is one of the most important biological mechanisms which involve the ability of microorganisms to accumulate heavy metals from contaminated site through metabolically mediated pathway [3].

Every year, severe global economic losses to agricultural crops are encountered due to plant diseases caused by more than sixty pathogens leading to the loss of 30% crop yield amounting 416 million US dollars [4]. Since agricultural fields due to the uncontrolled use of chemical pesticides and fertilizers are most contaminated, search for PGPR having potential of adsorbing heavy metals from agriculture field will have triple advantage of bioremediation, plant growth promotion and disease management [5]. Biocontrol through siderophore-mediated competition for iron have merged as a sustainable approach for integrated plant disease management [6–10].

Siderophores are also found to complex with heavy metals like cadmium, lead, nickel, arsenic (III, V), aluminium, magnesium zinc, copper, cobalt, and strontium other than iron [11, 12]. Under iron stress conditions, rhizobacteria produce siderophores that chelate the available iron and prevent the iron nutrition of respective phytopathogen [13] and there by restrict the proliferation and root colonization by phytopathogen. Siderophore producing rhizobacteria are also known to impart induced systemic resistance (ISRs) to the plants [14, 15] and suppressiveness to the soil [16] and have been implicated in the biocontrol of several plant diseases [17]. Siderophore based biological control agents (BCAs) are

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gaining commercial significance as they are safer, do not lead to biomagnification, their self-replication circumvents repeated application and target organisms do not develop pesticide resistance [6]. They also provide iron nutrition to the crops thereby promote the plant growth [18, 19].

The present work focuses on the antifungal activity of siderophore producing heavy metal resistant *Alcaligenes* sp. and *Pseudomonas aeruginosa* RZS3, against some common phytopathogenic fungi and bacterial strain.

Materials and Methods

Sources of Cultures

Two isolates were obtained from local soil and were labeled as *Alcaligenes* sp. *Pseudomonas aeruginosa* RZS3. Fungal cultures like *Aspergillus niger* NCIM 1025; responsible for causing crown rot and collar rot in groundnut and other crops, *Aspergillus flavus* NCIM 650; causing afla-root and aflatoxin production in groundnut, *Fusarium oxysporum* NCIM 1008; causing vascular wilt in radish, cucumber and onion, *Metazhizium anisophliae* NCIM 1311, and the *bacterial strain Pseudomonas solanacerum* NCIM 5103 were procured from National Center for Industrial Microorganisms [NCIM], NCL, Pune, India, *Alternaria alternata* IARI 715 was procured from Indian Agricultural Research Institute [IARI], New Delhi, India, *Cercospora arachichola* was isolated from infected groundnut. *Alcaligenes* sp., *Pseudomonas aeruginosa* RZS3 and *Pseudomonas solanacerum* were routinely maintained on nutrient agar and fungal cultures were maintained on potato dextrose agar (PDA). All the cultures were preserved at 4°C.

Heavy Metal Resistance

To obtain maximum heavy metal resistance level for *Alcaligenes* sp. and *Pseudomonas aeruginosa* RZS3 they were stepwise inoculated on nutrient agar plate with increasing grades of heavy metal concentrations of $MnCl_2$ and $NiCl_2$.

Screening for Siderophore Production

In order to screen siderophore production ability, *Alcaligenes* sp. and *Pseudomonas aeruginosa* RZS3 were inoculated into sterile succinic acid medium (SAM) containing (gl^{-1} in distilled water): K_2HPO_4, 6.0; KH_2PO_4, 3.0; $(NH_4)_2SO_4$, 1.0; $MgSO_4{\cdot}7H_2O$, 0.2; $C_4H_4Na_2O_4{\cdot}6H_2O$, 4.0; and pH.7, at 28 ± 2°C at 120 rpm for 24–48 h [20].

Partial Identification and 16 s rRNA Sequencing

The isolates were subjected to various biochemical tests as per the procedure and protocols of Bergey's manual of systematic bacteriology [21]. The pre-sterilized Hi-carbohydrate biochemical kits (KB 002 and KB 009, Hi Media, Mumbai, India) were used for biochemicals identification of the isolate. Further this partially identified culture was subjected to 16 s rRNA gene profile. Standard phenol–chloroform methods were used for genomic DNA extraction [22]. The 16S rRNA genes of the isolate were amplified by PCR [23] and sequenced directly on an automated DNA sequencer (ABI377) using the Big Dye terminator kit (Applied Biosystems) [24]. Results were compared with the public databases (NCBI) to determine the identity and homology of the isolate.

Siderophore Production, Detection and Estimation

Growth and siderophore production was carried out in 500 ml Erlenmeyer flask containing 100 ml of modified SAM [20]. For this purpose, *Alcaligenes* sp. and *Pseudomonas aeruginosa* RZS3 [6×10^6 cells ml^{-1}] were grown independently in SAM at 28 ± 2°C at 120 rpm for 24–48 h. After the incubation, cell density was measured at 620 nm by using double beam UV–Visible spectrophotometer [1240, Shimadzu, Japan]. The detection and estimation of siderophores was performed following the centrifugation at 15 min 5,000×*g* cm at 4°C and cell free supernatant was assayed for the presence of siderophore by using Chrome Azurol Sulphonate (CAS) test [25]. CAS shuttle assay [26, 27] was used to measure siderophores produced by the *Alcaligenes* sp. and *Pseudomonas aeruginosa* RZS3.

In Vitro Interaction with Phytopathogenic Fungi

In vitro phytopathogen suppression activity of siderophore and siderophoregenic culture preparations of *Alcaligenes* sp. and *Pseudomonas aeruginosa* RZS3 was directed against *Aspergillus niger*, *Aspergillus flavus*, *Fusarium oxysporum*, *Alternaria alternata*, *Cercospora arachichola*, *Metarhizium anisopliae* and *Pseudomonas solanacerum.* These strains are known to be common phytopathogens capable of causing major damages to the groundnut and other crops. In vitro antifungal activity was based on the principle of diffusion in which spore suspension [6×10^6 spores ml^{-1}] of above mentioned fungal sp. was separately mixed with molten PDA and poured in sterile petri plate. After hardening of medium, three wells each of about 10 mm, were bored in each plate and were separately added with 25, 50, 75 and 100 µl, each of culture broth [6×10^6 cell ml^{-1}], cell free supernatant and kitazin. While the antibacterial activity was checked by spreading the culture of *Pseudomonas solanacerum* [6×10^6 cells ml^{-1}] on the nutrient agar plates, three wells each of about 10 mm, were bored in each plate and were separately added with 25, 50, 75 and 100 µl, each

of culture broth [6×10^6 cell ml^{-1}], cell free supernatant and kitazin. Control was prepared by removing siderophore through the addition of 8 hydroxyquinone. Inoculated plates were kept for diffusion at 4°C for 15 min, incubated at 29°C for 48 h and were observed for the inhibition of fungal and bacterial growth. Antifungal and antibacterial potential of various preparations was determined by measuring the zone diameter of reduced fungal and bacterial growth. Zone diameter of more than 8 mm was considered as an indication of growth inhibition.

Determination of Minimum Inhibition Concentration (MIC)

In order to determine the MIC of the preparations, various preparations like culture broth [6×10^6 cells ml^{-1}], cell free supernatant and kitazin were separately taken in the range of 20–100 µl. Each preparation was separately added into PDA previously seeded with fungal pathogen [one fungus per plate]. While the antibacterial activity was checked by spreading the culture of *Pseudomonas solanacerum* [6×10^6 cells ml^{-1}] on the nutrient agar plates, these plates were allowed to diffuse at 4°C and incubated at 29 ± 1°C for 48 h.

Results and Discussion

Heavy Metal Resistance

Maximum metal resistance level for bacterial *Alcaligenes* sp. and *Pseudomonas aeruginosa* RZS3 were observed on nutrient agar with different concentrations of $MnCl_2$ and $NiCl_2$. *Pseudomonas aeruginosa* RZS3 showed resistant to $MnCl_2$ salt up till 3 mg during step by step repeated culturing on nutrient agar. Similarly resistance of *Alcaligenes* sp. was obtained upto 1 mg of $NiCl_2$. Most bacterial strain accumulates metal by employing physico-chemical mechanisms and transport system of varying specificity. However, both essential and non-essential metals in concentrations, higher than optimal level, prove toxic to organisms. Under such conditions, these organisms may activate and adapt a mechanism of detoxification to ensure survival. In this study we were successful in developing Ni and Mn resistant strains by step-by-step repeated culture and selection on the medium containing increasing concentration of Ni and Mn.

Partial Identification and 16 s rRNA Sequencing

Preliminary phenotypic characterization showed that the isolates were a Gram-negative straight, motile rod, presented a fermentative metabolism on a wide range of sugars.

Table 1 Preliminary identification of *Alcaligenes* sp. and *Pseudomonas aeruginosa* RZS3

Sl no.	Characteristics	Results	
		Alcaligenes sp.	*Pseudomonas aeruginosa* RZS3
1	Gram staining	Negative	Negative
3	Phosphate solubilization	Negative	Positive
4	Detection of siderophore	Positive	Positive
5	Motility	Motile	Motile
6	Starch hydrolysis	Negative	Positive
7	Utilization of carbohydrates		
	a. Glucose	Positive	Positive
	b. Fructose	Negative	Negative
	c. Sucrose	Negative	Negative
	d. Raffinose	Negative	Negative
	e. L-Arrabinose	Positive	Positive
	f. Mannose	Positive	Positive
	g. Ribose	Positive	Positive
12	Citrate-utilization	Positive	Positive
13	Nitrate reduction	Negative	Negative

(Table 1) and synthesized a fluorescent pigments when grown on nutrient agar. The isolates were named as *Alcaligenes* sp. and *Pseudomonas aeruginosa* RZS3. 16S ribosomal RNA partial gene sequencing of this isolates showed close i.e. 98 and 99% relationship with *Alcaligenes* sp. STC1 and *Pseudomonas aeruginosa* RZS3 SH-94B, respectively, therefore, this isolates were named as *Alcaligenes* sp. STC1 and *Pseudomonas aeruginosa* RZS3 SH-94B.

Screening for Siderophore and Siderophore Production and Detection

In the shake flask studies, change in the color of SAM from colorless to fluorescent green after 24 h, indicated siderophore production. Production of siderophore was confirmed by CAS test, where addition of CAS to cell free supernatant changed the blue color of CAS to orange while no color change occurred in control (un-inoculated). Change in the color of CAS reagent was due to the fact that siderophore present in the supernatant chelates the iron from CAS reagent and results in color change from blue to orange red [25–27].

Alcaligenes sp. excreted highest amount (92.61%) of siderophore while *Pseudomonas aeruginosa* RZS3 produced less amount (43.22%) of siderophore.

Siderophores produced by rhizobacteria chelate available iron and therefore create artificial shortage of iron to the respective phytopathogens thereby limiting their growth [13]. In vitro phytopathogen suppression by *Alcaligenes* sp., *Pseudomonas aeruginosa* RZS3 indicated their biocontrol potential. Both siderophore rich culture broth as well as cell

free supernatant were found to inhibit the growth of phytopathogenic fungi namely *A. niger*, *A. flavus*, *F. oxysporum*, *A. alternata*, *C. arachichola*, *M. anisopliae* and *P. solanacereum*. Control preparation [free of any siderophore activity] did not inhibit the growth of any of the fungal sp. under study. These results suggested that, cell free culture supernatant as well as siderophore rich culture broth have the biocontrol potential against phytopathogenic fungi and *P. solanacereum*. However, siderophore rich culture broth (Tables 2, 3 and Fig. 1) proved to be potent inhibitor of fungal pathogens than cell free culture supernatant (Tables 2, 3 and Fig. 2) and the chemical fungicide kitazin (Table 4, Fig. 3). The presence of siderophoregenic rhizobacteria around root zone of plants is known to protect the plant from phytopathogen infestations by preventing its iron nutrition [10, 28, 29].

Determination of MIC

As depicted in Table 2 and 3, the MIC of culture broth containing siderophoregenic *Alcaligenes* sp. and *Pseudomonas aeruginosa* RZS3, respectively, was 25 µl for *A. niger*, *F. oxysporum*, *A. flavus*, *C. arachichola* and *P. solanacerum* 75 µl for *A. alternata*. The MIC of cell free supernatant was 75 µl for *A. niger*, *A. alternata*, *A. flavus*, *F. oxysporum*, *C. arachichola* and *P. solanacerum.* As per the Table 4 the MIC of kitazin (fungicide) was 75 µl for *A. niger* and *A. flavus* while 100 µl for *F. oxysporum*, *A. alternata*, *C. arachichola*, *M. anisopliae* and *P. solanacerum*.

Antifungal Activity Against Phytopathogenic Fungi

Siderophores produced by heavy metal resistant isolates have been implicated in the biocontrol of several diseases, like vascular wilts caused by *F. oxysporum* and stem rot of pea nut caused by *Rhizoctonia solani* [17]. It have been reported that siderophore producing *Pseudomonas aeruginosa* was capable of inhibiting the growth of *A. niger*, *A. flavus*, *F. oxysporum* and *A. alternata* [30]. It have been also reported that hydroxamate type of siderophore producing *A. chrococcum* RRLJ 203 inhibited the growth of *F. oxysporum* and other phytopathogenic fungi [31].

Table 2 MIC of siderophore based preparation using *Alcaligenes* sp. against some common phytopathogenic fungi and bacterial strain

Preparation	Amount (µl)	Diameter of zone of inhibition (mm)						
		A. niger NCIM 1025	*A. flavus* NCIM 650	*F. oxysporum* NCIM 1281	*A. alternata* IARI 715	*C. arachichola arachichola*	*M. anisopliae* NCIM 1311	*P. solanacerum* NCIM 5103
Culture broth (6×10^6 cell ml^{-1})	25	24.0	27.0	23.0	13.0	40.0	30.0	18.0
	50	27.0	35.0	28.0	15.0	35.0	35.0	20.0
	75	30.0	30.0	29.0	27.0	33.0	40.0	22.0
	100	35.0	32.0	30.0	29.0	45.0	45.0	24.0
Culture	25	22.0	35.0	20.0	19.0	29.0	30.0	11.0
Supernatant	50	25.0	38.0	22.0	22.0	35.0	35.0	16.0
	75	40.0	45.0	24.0	25.0	32.0	32.0	15.0
	100	40.0	47.0	25.0	28.0	39.0	39.0	19.0

Table 3 MIC of siderophore based preparation using *Pseudomonas aeruginosa* RZS3 against some common phytopathogenic fungi and bacterial strain

Preparation	Amount (µl)	Diameter of zone of inhibition (mm)						
		A. niger NCIM 1025	*A. flavus* NCIM 650	*F. oxysporum* NCIM 1281	*A. alternata* IARI 715	*C. arachichola*	*M. anisopliae* NCIM 1311	*P. solanacerum* NCIM 5103
Culture broth (6×10^6 cell ml^{-1})	25	23.0	26.0	25.0	15.0	28.0	23.0	16.0
	50	26.0	28.0	32.0	18.0	32.0	25.0	18.0
	75	35.0	25.0	35.0	19.0	37.0	27.0	20.0
	100	37.0	32.0	40.0	17.0	40.0	30.0	22.0
Culture	25	25.0	20.0	32.0	15.0	28.0	20.0	11.0
Supernatant	50	25.0	22.0	35.0	18.0	32.0	22.0	14.0
	75	28.0	35.0	38.0	19.0	37.0	24.0	16.0
	100	29.0	42.0	40.0	17.0	40.0	25.0	18.0

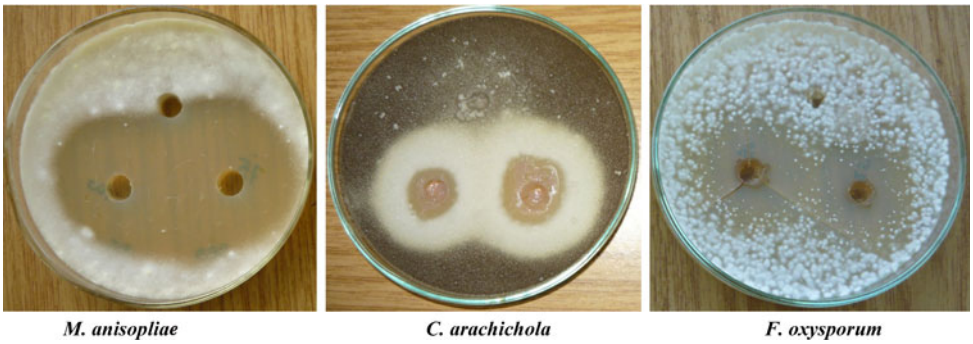

Fig. 1 Antifungal activity of siderophore rich broth of *Alcaligenes* sp.

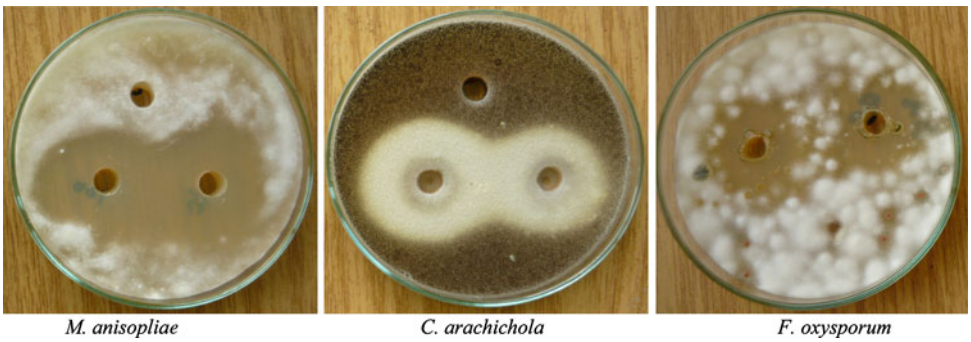

Fig. 2 Antifungal activity of siderophore rich supernatant of *Alcaligenes* sp.

Table 4 MIC of Kitazin (fungicide) against some common phytopathogenic fungi and bacterial strain

Preparation	Amount (μl)	Diameter of zone of inhibition (mm)						
		A. niger NCIM 1025	*A. flavus* NCIM 650	*F. oxysporum* NCIM 1281	*A. alternata* IARI 715	*Cercospora arachichola*	*M. anisopliae* NCIM 1311	*P. solanacerum* NCIM 5103
Kitazin	25	0.7	1.5	15.0	20.0	15.0	18.0	15.0
	50	0.7	1.5	20.0	25.0	15.0	20.0	12.0
	75	1.0	2.0	24.0	30.0	15.0	22.0	11.0
	100	1.0	2.5	25.0	35.0	15.0	22.0	11.0

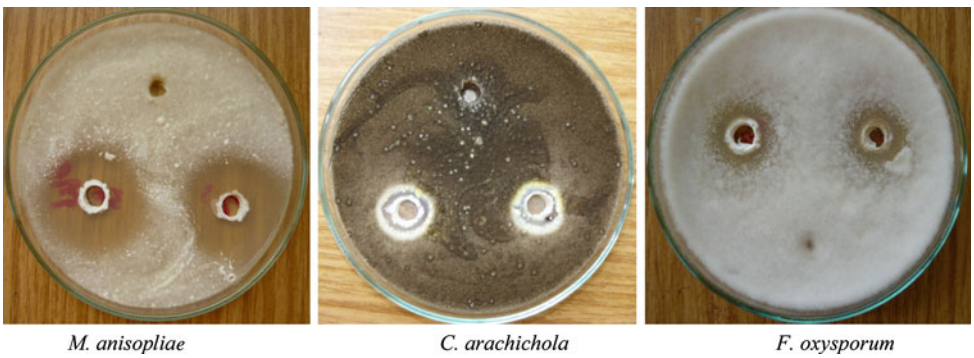

Fig. 3 Antifungal activity of chemical fungicide (kitazin)

The siderophoregenic culture broth proved to be potent inhibitor of fungal pathogens than kitazin and cell free culture supernatant indicating the role of other secondary metabolites along with siderophores in the growth inhibition of pathogenic fungi.

Evaluation of Safety for Useful Soil Rhizobia

To be an ideal antagonist, the BCA should be effective against a wide range of pathogens but should not harm the useful soil rhizobia. It have been reported that siderophore

producing *P. aeruginosa* did not cause the growth inhibition of *A. vinelandii*, *R. meliotii* and *B. japonicum* [30]. Rhizobacteria are known to possesses iron regulated outer membrane protein receptors on their cell surface that transport ferric iron complex to the respective cognate membrane [10] and are not deprived of their iron nutrition by the siderophore producing rhizobacteria and thus their growth is not affected [32]. These results provide evidence for eco-friendly role of siderophoregenic *Alcaligenes* sp. and *Pseudomonas aeruginosa* RZS3 as a potent BCA.

Conclusion

Siderophore rich broth and supernatant are ecofriendly biocontrol agents and have greater antifungal potential than chemical fungicide kitazin. Research into the mechanisms of plant growth promotion by *Alcaligenes* sp. and *Pseudomonas aeruginosa* RZS3 have provided a greater understanding of the multiple facets of disease suppression by these biocontrol agents.

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