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Research Article

In Vitro Antagonism of *Pseudomonas aeruginosa Al98* Towards *Sclerotium rolfsii*, a Causative Agent of Stem Rot Disease of Groundnut

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Abstract

Stem rot disease of groundnut caused by *Sclerotium rolfsii* is difficult to control by conventional means such as fungicides; therefore, the naturally available antagonist present is tested for control. In present study, 11 *Pseudomonas* strains isolated from rhizospheric soil were screened for their biocontrol activity against *S. rolfsii* under *in vitro* conditions. One of the *Pseudomonas* strain, identified as *Pseudomonas aeruginosa AL98*, was selected for further studies because of their ability to inhibit the mycelia growth of the pathogen significantly. In dual cultures, the *Pseudomonas aeruginosa AL98* inhibited the *Sclerotium rolfsii* up to 94 % in terms of dry weight. *Pseudomonas aeruginosa AL98* produced catecholate type of siderophore which affect *Sclerotium rolfsii* growth *in vitro*.

INTRODUCTION:

Rhizospheric bacteria serve as excellent agents to control soil-borne plant pathogens. Bacterial species like *Bacillus*, *Pseudomonas*, *Serratia* and *Arthrobacter* have been proved in controlling the fungal diseases. Bacteria identified as plant growth promoting rhizobacteria and biocontrol strains often belong to the following genera. (i) *Bacillus* (Nair *et al.*, 2002, Shadi Ray *et al.*, 2012 and Selseleh-zakeri *et al.*, 2015), (ii) *Streptomyces* (Adb-Allah, 2001) and (iii) *Pseudomonas* (Mark *et al.*, 2006; Dalvi and Rakh, 2017).

Pseudomonas spp received great attention as biocontrol agent because of their catabolic versatility, excellent root-colonizing abilities and production of broad range antifungal metabolites such as 2,4-diacetylphloroglucinoal (DAPG), pyoluteorin, pyrrolnitrin and phenazines (Chin-A-Woeng *et al.*, 2001; Raaijmaker *et al.*, 2002). The mechanisms through which *Pseudomonas* spp. control plant diseases involve (i) competition for niches and nutrients, (ii) antibiosis, (iii) predation, and (iv) induction of plant defense responses.

Sclerotium rolfsii Sacc is a soil-borne plant pathogen of worldwide importance with a very extensive host range including more than 500 plants species. Most *S. rolfsii* diseases have been reported on dicotyledonous hosts, but with several monocotyledonous species also being infected. *Sclerotium rolfsii* is especially severe on legumes, Solanaceous crops, cucurbits and other vegetables grown in rotation with beans (Tu, 1978; Wydra 1996). The traditional agricultural practice to control the phytopathogen is by using variety of Fungicides e.g. Bavistin, Captan etc. but a sever disadvantage of the traditional method, that it is not effective to check the *Sclerotium* during the cropping duration (90- 100 days) and is not eco-friendly. Hence, as an alternative attempt has been made to give an eco-friendly strategy for the control of *Sclerotium* during this work. The objective of the current investigation was to isolate an efficient biocontrol agent, *Pseudomonas*

spp, from rhizospheric soil of healthy plants such as Neem and evaluate its potential in controlling the soil-borne pathogen, *Sclerotium rolfsii*, causing stem rot of groundnut.

MATERIALS AND METHODS:

Fungal culture of *Sclerotium rolfsii* was obtained from the Department of Plant Pathology, Marathwada Agricultural University, Parbhani (Maharashtra), India. The fungal culture was maintained on Potato Dextrose Agar medium at 30°C.

Isolation of *Pseudomonas spp*. from rhizospheric soil:

Rhizospheric soil from different healthy plants such as soybean, neem, groundnut, tur etc. were collected in poly-ethylene bags and brought to the research laboratory. A 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* spp, 1ml of this nutrient broth was transferred to selective enrichment media, 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 (Brown and Lowbury, 1965) 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 against the phytopathogen for biocontrol ability. All the isolates were tentatively named during this research to avoid confusion.

Screening for Potential Biocontrol agents against phytopathogen:

All the isolates were screened for potential antagonistic activity against pathogenic fungi *S.* on King's B agar (Ran, *et al.*, 2003) using dual culture technique (Rangeshwaran and Prasad, 2000). An agar disc (5 mm dia.) was cut from an actively growing (96 h) phytopathogen, *S. rolfsii* culture 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 bacterial isolates (each) was placed opposite to the fungal disc. Plates inoculated with phytopathogen and without bacteria were used as control. 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 and percentage inhibition calculated by the following equation (Riungu *et al.*, 2008).

$$\text{Inhibition (\%)} = \frac{\text{Colony diameter of Pathogen - Colony diameter of Pathogen alone (Control) + Antagonist}}{\text{Colony diameter of Pathogen alone}} \times 100$$

Identification of Biocontrol agent:

An efficient biocontrol agent obtained from screening was identified according to Bergey's Manual of Systematic Bacteriology (1984) by using cultural, biochemical characteristics as well as 16s rRNA sequencing.16s rRNA sequencing of culture was carried out at Agharkar Research Institute (ARI) Pune, Maharashtra.

***In vitro* Characterization of biocontrol features:**

To investigate the biocontrol mechanism, the efficient rhizospheric isolate was tested for siderophore assay.

Siderophore Analysis:

Inoculum Preparation

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

Culture and Cultivation

Siderophore production was studied using modified Succinate medium (Meyer and Abdullah, 1978) consisting following components (g/l) Succinic acid (4), K_2HPO_4 (6), KH_2PO_4 (3), $(NH_4)_2SO_4$ (1), $MgSO_4$ (0.2), and pH (7.0). 0.1ml of inoculum were separately inoculated in 250 ml Erlenmeyer flask containing Succinate medium and then incubated on rotatory shaker incubator for 48 h at 28^{o}C. A supernatant was harvested by centrifuging the culture at 10,000 rpm in cooling centrifuge at 4^{o}C for 10 minute.

Siderophore Detection:

Qualitative Detection of Siderophore

Qualitatively siderophore production was detected as per (Schwyn and Neilands, 1987) using Universal Chemical Assay (CAS).

Here equal volume of supernatant and CAS reagent was mixed in a clean test tube and observed for color change as compare to control or reference.

Quantitative Detection of Siderophore

Quantitatively siderophores in culture filtrate were detected as per (Payne, 1994) where 0.5 ml of cell free culture filtrate was mixed with 0.5 ml of CAS solution. A reference was prepared using, uninoculated succinate medium (used for siderophore production by *Pseudomonas*). Both the test and reference were read at 630 nm and % siderophore units (% decolorization) in the culture filtrate were calculated.

$$\% \text{ Siderophore Units} = \frac{Ar - As}{Ar} \times 100$$

Where,

Ar = Absorbance of reference at 630 nm.
As = Absorbance of test sample at 630 nm.

Detection of Siderophore type

Type of siderophore i.e. either Hydroxamate or Catecholate, was determined by Csaky assay (Csaky, 1948) for hydroxamate and Arnow's assay (Arnow, 1937) for Catecholate/Phenolates.

Csaky Assay for detection of Hydroxamate type siderophores

The siderophore solution or supernatant (1 ml) was hydrolyzed with 1 ml of (6 N) H_2SO_4 in boiling water bath for 6 h or at 130 ^oC for 30 min. The solution was then buffered by adding 3 ml Sodium acetate solution, further 1 ml of 0.5 ml Iodine solution was added. After 3-5 min. excess Iodine was destroyed with 1 ml Sodium arsenate solution. Finally 1 ml α-Naphthalamine solution was added, allowed to react for 20-30 min for development of color.

Arnow's Assay for Catecholate-type Siderophores

To 1 ml culture supernatant, 1 ml 0.5 M HCl was added and mixed properly, further 1 ml of Nitrite-Molybdate reagent (prepared by dissolving 10 gm Sodium nitrite and 10 g Sodium molybdate in 100 ml distilled water) and 1 ml 1 M NaOH was added. This mixture was allowed to react for 5 minutes for the reaction to fully occur. Absorbance was measured at 510 nm. Uninoculated media instead of supernatant used as blank. Catecholate groups can be detected because they form a yellow color in Nitrous acid, which turns pink-red when excess NaOH was present. A control (either a culture grown in high iron or uninoculated media) will remain colorless with the addition of reagents.

RESULT AND DISCUSSION:

Isolation of *Pseudomonas spp*. from rhizospheric soil:

During this research work, 11 *Pseudomonas spp* were isolated from rhizospheric soil of different healthy plants such as Soybean, Neem, Groundnut, Tur etc. All the rhizospheric isolates were tentatively named as in Table 1 and maintained on Nutrient Agar slants for further screening.

Screening for Potential Biocontrol agents against phytopathogen:

It was observed that *Pseudomonas* isolate SMD found as an efficient antagonist against *S. rolfsii* in dual culture technique (Photo Plate 1) while none of the other *Pseudomonas isolates* found efficient (Table 1).

It was revealed that culture SMD was able to inhibit *Sclerotium rolfsii* (94 %). Our results when compared with the results earlier reported (Kishore *et al.*, 2005), for control of *Sclerotium rolfsii* with *Pseudomonas aeruginosa* in dual culture. It was found that our results with *Pseudomonas* far better than the above-mentioned results because there was only 32-74 % inhibition recorded whereas in our results 94 % inhibition was recorded for *S. rolfsii*. Similar kind of results were also observed by Dalvi and Rakh, (2017), where in dual culture technique, the *Pseudomonas cf. monteilii 9* inhibited the *Sclerotium rolfsii* (94 %), in terms of dry weight.

Identification of Biocontrol agent:

The efficient SMD was identified by 16S rDNA gene sequencing. The sequence was edited

and aligned with sequence in the public domain GenBank by BLAST Programm which showed 98 % similarity with accession number AJ249451. The biocontrol agents exert a protective effect on roots through antagonism towards phytopathogenic

***In vitro* Characterization of biocontrol features:**

fungi. Two major mechanisms have been proposed to explain the suppressive and antagonistic effects of *Pseudomonads* in particular i.e. either the phytopathogen is inhibited by competition for iron, as availability of Fe^{+++} in soil is low (10^{-17} M). OR secondly *Pseudomonas* inhibit the pathogens by producing secondary metabolites with antibiotic activity e.g. Phenazine, Pyrrolnitrin, Phenazines, 2,4-diacetylphloroglucinol and cyanides (Winding *et al*., 2004). Nevertheless, disease suppression is a multifunctional attributes, hence for understanding the mechanism of action shown by the strains under study variety of experiments were carried out during this research work.

To investigate the biocontrol mechanism of the selected strain namely, *Pseudomonas aeruginosa AL98* was tested for production of siderophore.

Siderophore Analysis:

Inoculum of pseudomonas culture was inoculated in modified Succinate medium (Meyer and Abdullah, 1978) and incubated on rotary shaking incubator at 28 ^{o}C for 48 h.

Siderophore Detection:

Qualitative Detection of Siderophore:

Pseudomonas aeruginosa AL98 gave instant color change of CAS reagent from blue to classical golden orange when tested qualitatively by Universal CAS assay which shows production of siderophore (Photo plate 2).

Quantitative Detection of Siderophore:

A supernatant of the culture was harvested after 12, 24, 36 and 48 h by centrifuging the culture at 10,000 rpm in cooling centrifuge. Quantitatively siderophores in supernatant was detected as per Payne, (1994) where 0.5 ml of culture filtrate was mixed with 0.5 ml of CAS solution. A reference was prepared using, uninoculated Succinate medium. Absorbance of both the test and reference were read at 630 nm and % siderophore units in the culture filtrate were calculated. *Pseudomonas aeruginosa AL98* gave instant color change of CAS reagent from blue to classical golden orange and produced1. maximum siderophore whose absorbance was read at 630 nm. % siderophore unit of culture was calculated after 12, 24, 36 and 48 h (Graph 1).

In the time course of siderophore production, maximum siderophore secretions by *Pseudomonas aeruginosa AL98* (89.89%) was recorded after 36 h. Siderophore being a secondary metabolite produced under iron stress condition hence maximum siderophore was recorded 36 h. thereafter, re-incubation showed decline in the % siderophore. Here for siderophore production the batch needs to be harvested after 36 h. for maximum recovery of siderophore.

Detection of Siderophore type

For determination of the type of iron chelating groups in siderophores of *Pseudomonas aeruginosa AL98*, the culture supernatant was subjected to Csaky's assay (Csaky, 1948) and Arnow's assay (Arnow, 1937).

Pseudomonas aeruginosa AL98 fails to produce instant wine-red color after addition of α-naphthalamine indicating absence of hydroxymate type of siderophore (Photo plate 3). While in Arnow's assay, it gave yellow color after addition of sodium molybdate reagent indicating presence of catecholate type of group but turn red after addition of sodium hydroxide (Photo plate 4).

Siderophore produced by certain strains of fluorescent *Pseudomonas* spp. have been reported to link in suppression of soil-borne plant diseases (Bashan and de-Bashan, 2005). It has been suggested that siderophores act antagonistically by sequestering iron from the environment, restricting growth of the pathogen. Convincing evidence for the involvement of siderophores in disease suppression is readily available (Bashan and de-Bashan, 2005). For example, a mutant strain of *P. putida* that overproduces siderophores has been shown to be more effective than the wild bacterium in controlling the pathogenic fungus *Fusarium oxysporium* in tomato. Many wild strains that lose their siderophore trait also lose biological control activity. *Pseudomonas aeruginosa AL98* produced only catecholate type of siderophore which playing a vital role in biocontrol of *S. rolfsii.* Also, Dalvi and Rakh (2017) displayed that *Pseudomonas cf. monteilii 9* produce siderophore which was responsible for suppression of *Sclerotium rolfsii* in vitro

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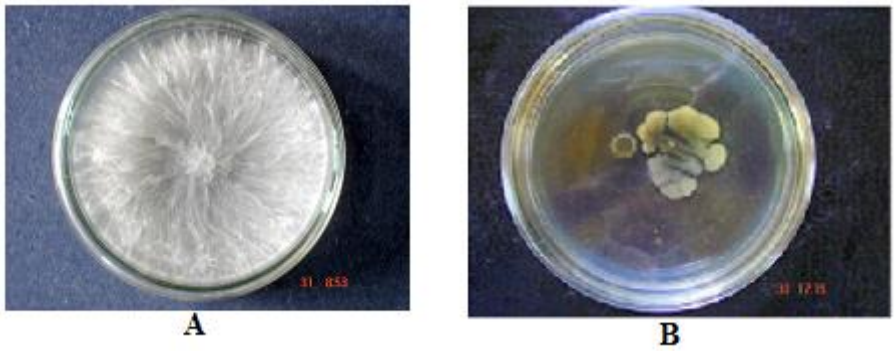

Photo Plate 1: Dual Culture technique of SMD against *Sclerotium rolfsii*
A) Control of *Sclerotium rolfsii* B) SMD culture with *Sclerotium rolfsii*

Table 1: *In vitro* screening for potential biocontrol agents against *Sclerotium rolfsii*

Strain	*Sclerotium rolfsii*
SMD	**4**
CA/RT	2
NM/S1/CA	0
NM/S2/CA	0
NM/S6/CA	0
NM/S8/CA	0
CA/RT	2
CA/RG	3
AVM 1	1
AVM 3	3
SBC-B	3

Each number is mean of three replicates. 0- none, 1= inhibition zone 1-25 %, 2= inhibition zone 26-50 %, 3= inhibition zone 51-75 %, 4= inhibition zone 76-100 %.

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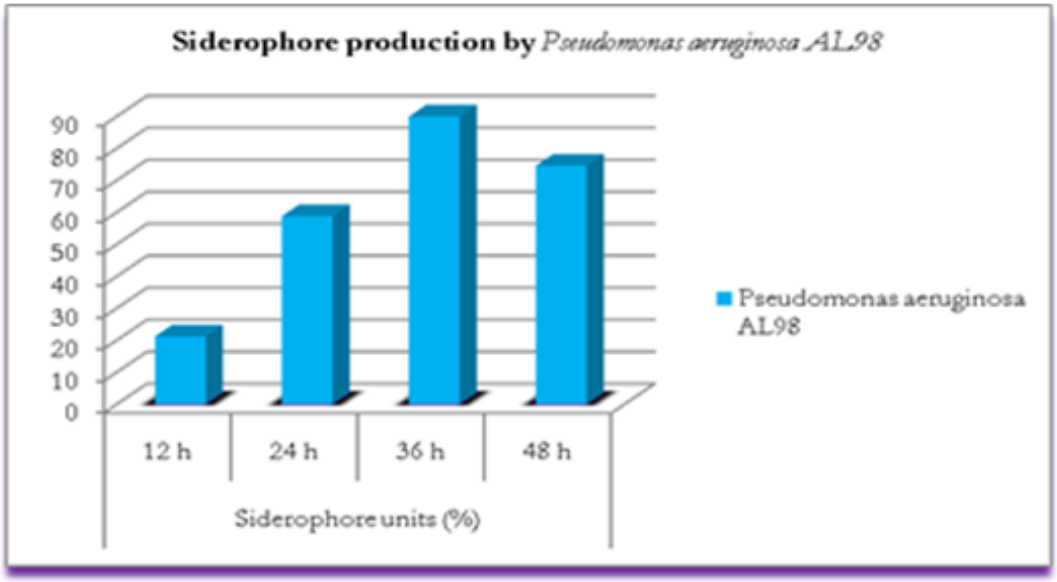
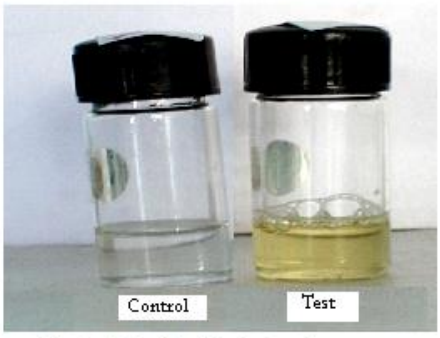
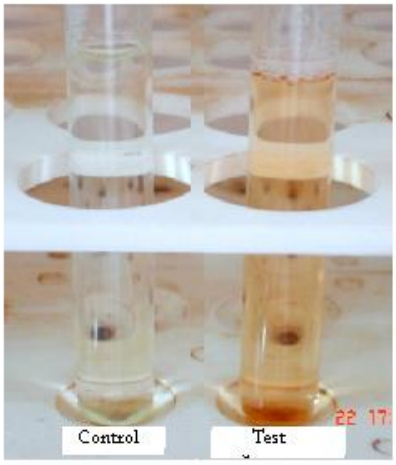
Photo Plate 2: Liquid CAS Assay for Siderophore Production
A) Test B) Control



Graph 1: Siderophore productions by *Pseudomonas aeruginosa AL98* with respect to time



Photoplate 3:- Csaky's Assay



Photoplate 4: Arnow's Assay

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