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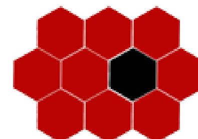

ORIGINAL PAPER

OPEN ACCESS

Evaluation of quorum sensing in biocontrol and plant growth promoting properties of *Pseudomonas* sp.

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Abstract

Background: Bacteria communicate with each other via chemical signals to coordinate expression of specific genes in a cell density dependent fashion a phenomenon called quorum sensing and response. *Pseudomonads* are ubiquitous bacteria that are common inhabitants of rhizosphere; these stimulate plant growth by facilitating either uptake of nutrients from soil or producing certain plant growth promoting substances. Besides, these bacteria also prevent proliferation of phytopathogens and thereby support plant growth.
Challenges: The present study deals with the quorum sensing mechanism which influences the antifungal activity of *Pseudomonas aeruginosa* and *Pseudomonas fluorescens* strains against the *Rhizoctonia solani*. The antifungal activity of the *Pseudomonas aeruginosa* and its extract was tested. The plant growth promoting ability was then analyzed and compared. Seed germination assay was also performed for proving quorum sensing.
Conclusion: Quorum Sensing in Biocontrol and Plant Growth Promoting Properties of *Pseudomonas* sp. was evaluated and found to be satisfactory.

Keywords: Quorum sensing, Antagonism, Seed germination assay, Biocontrol.

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INTRODUCTION

Plants are considered as the source of foods, nutrients, valuable medicinal products etc., food products are considered as the major outcome of plants. Next to China, India is considered to be the largest producer of vegetables. The major crops cultivated in India includes rice, tomato, etc., most of the cereals including rice is affected by various pathogens including bacteria, fungi and virus (Anil *et al.,* 2009). Out of them, Sheath Blight (ShB) disease of rice is the major yield affecting disease caused by the phytopathogenic fungi, *Rhizoctonia solani* (Saikia *et al.,* 2006). They yield loss due to ShB disease was up to 50% globally and in India it was up to 69% (Tang *et al*., 2007). Since the sclerotia of the fungi can persist in soil for several years, it is difficult to control the disease by commercial methods.

Rhizoctonia solani is a multi targeted fungi which can also infect crops like Cabbage, Tomato, Maize, Potato, etc., (FFTC, 2004). Currently chemical fungicides and antibiotics were used for the management of Sheath Blight disease (Dev and Mary, 1986). Since the overuses of chemicals are having harmful effects over humans, an alternate biological control has been attracting attention (Orie and Makoto, 1996). Biological control is the efficient tool for management of various plant diseases (Mathivanan *et al*., 2006). Several attempts were made in India and other countries to control ShB of rice using Biocontrol agents (Mew and Rosales, 1986; Fan *et al.,* 2004; Mathivanan *et al.,* 2005).

Plant Growth Promoting Rhizobacteria (PGPR) are widely used Biocontrol agents which controls plant diseases and also promotes plant growth (Prasanna *et al*, 2010). Fluorescent Pseudomonads are PGPRs known to inhibit several plant pathogenic fungi. The applicability of Pseudomonads as biocontrol agents has been gaining wide attention because of the production of various secondary metabolites. Production of lytic enzymes, siderophores, hydrogen cyanide and biosynthesis of antibiotics, competition for substrates in the rhizosphere and induced systematic resistance (ISR) are the major mechanisms proposed for antagonistic activities of FPs against plant pathogens. (Handelsman and Stabb, 1997).

Fluorescent pseudomonads produce yellow-green, fluorescent siderophores which specifically recognize and sequester the limited supply of iron in the rhizosphere and thereby reduce the availability of this trace element for the growth of the pathogen. Indole-3-acetic acid, also known as IAA, is probably the most important plant auxin.

Pseudomonas aeruginosa is known to be antagonistic for *R. solani* (Saikia *et al.,* 2006). It also helps in the enhancement of plant growth. Some strains of *Pseudomonas fluorescens* are found to be having antagonistic potential towards *R. solani.* Quorum sensing has been well observed in the *P. aeruginosa* in the case of pathogenicity, Biofilm formation, etc., (Bassler and Lostick, 2006). Quorum sensing is the decision making process used by decentralized group of bacteria to coordinate behavior. Many species of bacteria use quorum sensing to coordinate their gene expression according to the local density of their population. Similarly, some social insects use quorum sensing to make collective decisions about, where to nest. In addition to its function in biological systems, quorum sensing has several useful applications for computing and robotics.

The present study was aimed at the screening of the antagonistic potential of *P. aeruginosa* and *P. fluorescens* against *R. solani.* The role of Quorum sensing between the two bacteria was analyzed when cultured with *R. solani.*

MATERIALS AND METHODS

Selection and culture of organisms: *Pseudomonas fluorescens* and *Pseudomonas aeruginosa* were collected and inoculated in to KB medium for further studies. *R. solani* was cultured and maintained in Potato Dextrose Agar (PDA).

Determination of antagonistic activity of *P. aeruginosa:*

Plate assay (Dual culture): Two PDA plates were taken and placed *R. solani* discs at the centre of each plate. Adjacent to the disc streaked the *P. aeruginosa* in first plate and *P. fluorescens* in second plate. The plates were sealed, labeled and incubated for two days. After incubation the plates were observed for the growth of fungal mycelium.

Flask assay (Dual flask assay): Two experimental setups were prepared with two conical flasks connected through a rubber tube. 25 ml of LB broth was filled in one flask and PDA broth on another flask. In the first setup, *P. aeruginosa* was inoculated in LB broth and *R. solani* on PDA broth which was considered as test. In the second setup, LB broth was uninoculated and PDA was inoculated with *R. solani* which was considered as

control. The apparatus was sealed tightly and was incubated for 2 days. After incubation the PDA broth was filtered and the mycelial dry weight was weighed (Dennis and Webster, 1971).

Dual Bottom Plate assay: Dual bottom assay was performed by the method followed by Dennis and Webster, 1971. Two bottom plates kept closed tightly in which LB agar on one lid and PDA on another lid. In control LB was uninoculated and PDA was inoculated with *R. solani*. In test, LB was inoculated with *P. aeruginosa* and PDA with *R. solani*.

Plant growth promoting studies:

Autoinducing metabolites: The *P. aeruginosa* was allowed to grow on Auto Inducing Metabolite (AIM) production medium (Vasudevan *et al.,* 2002) for 24 hrs under shaken condition. The cell debris was removed by centrifugation and the supernatant was further processed for the extraction of AIM using ethyl acetate with the help of rotary evaporator. The extracted AIM was dissolved in acetone for further processing.

Poison plate method: PDA plates with three different concentrations (10µg/ml, 20µg/ml, and 40µg/ml) of the drugs (AIM extract) were prepared. Then the sclerotium of *R. solani* was placed at the centre of each PDA plate with different concentrations of drugs. The plates were incubated for 2 days and the fungal growth was observed.

Volatile metabolites (HCN): KB medium supplemented with 4.4g/l of glycine was used to detect the production of Hydrogen cyanide (HCN). Filter paper strips saturated with 0.5% picric acid in 2% aqueous sodium carbonate solution were placed on the lid of the KB plates spread with *Pseudomonas aeruginosa*. The plates were sealed and incubated for two days. The filter paper strips were then observed for any colour change if happens due to the production of HCN (Lorck, 1948).

Siderophores: *P. aeruginosa* was screened for the production of iron scavenging protein, siderophore by streaked the bacteria onto the King's B media with and without (50 mg L^{-1}) $FeCl_3$ and incubated at 30°C for 48 h. The presence of the fluorescent pigment around the bacterial colony will confirm the production of siderophores.

Indole acetic acid: IAA production was detected by the modified method as described by Brick et al. (1991). Development of pink colour indicates IAA production.

Phosphate Solubilization: The organism was screened for its phosphate solubilizing ability by streaking *P. aeruginosa* over the Pikovskaya agar medium (Pikovskaya, 1948) and incubated for 3 days. Formation of clear zones will indicate the solubiliztion of Phosphate.

Production of Ammonia: Since ammonia is also an essential element for plant growth, the ammonia production was screened. The bacteria was inoculated in 10 ml of peptone water and incubated for 3 days. After incubation Nessler's reagent (0.5 ml) was added to the tube. Development of brown to yellow colour was a positive test for ammonia production (Cappuccino and Sherman, 1992).

Effect of *P. aeruginosa* on fungal protein production:

Co-culture: Potato dextrose broth was prepared for 25 ml and inoculated with *P. aeruginosa* and *R. solani*. Another 25 ml of Potato dextrose broth was taken and inoculated with *R. solani* alone which was kept as control while the former was considered as test. After 2 days incubation the fungal mycelium was collected by filtering the media through filter paper. The fungal mycelium was grinded with the help of 1 M Phosphate buffer. The grinded mixture was then centrifuged and collected the supernatant.

SDS PAGE: The supernatant of the control and test were allowed to run on SDS PAGE and the difference in the protein profile of test and control were observed using silver nitrate staining.

Determination of quorum sensing:

Plate assay: The plate assay for determining the quorum sensing using organisms was done by using PDA plates which was inoculated with *R. solani* at one side and *P. aeruginosa* and *P. fluorescence* on another side. The plates were then observed for inhibition of fungal growth after two days incubation. The plate assay using metabolites was similar to the plate assay using organism. But the *P. aeruginosa* organism was replaced with AIM extract of *P. aeruginosa* was poured on the well on the same place. The plates were then observed for inhibition of fungal growth after two days incubation.

Flask assay: Three flasks were prepared with 50 ml of Kings B broth in each. *P. fluorescence* was added to the first flask whereas *P. aeruginosa* in second and both the *P. aeruginosa* and *P. fluorescence* in third flask respectively. The fluorescence was observed under UV light after 24 hours of incubation.

Seed germination assay: Seeds of tomato, brinjal and lady's finger were surface sterilized with sodium hypo chloride and washed with distilled water followed by blot drying. Each type of seeds were immersed in the following combination of liquid cultures for 40 minutes each (Prashanth and Mathivanan, 2009). Three seeds were placed in each petridishes with moist tissue paper and the plates were maintained in a growth chamber. The germination was observed after 4 days for control, treatment with *R. solani,* treatment with *P. aeruginosa* and *R. solani,* treatment with *P. fluorescens* and *R. solani* and treatment with *P. aeruginosa*, *P. fluorescens* and *R. solani.*

RESULTS AND DISCUSSION

In agriculture, the excessive use of pesticides has caused many harmful effects to the consumers like human beings. To minimize the pesticide or to avoid pesticide usage in agriculture field, the biological control is the effective alternative tool in controlling several plant diseases. (Mathivanan *et al*., 2006). Quorum sensing is a type of cell signaling occurs between groups of bacteria (Pesci *et al.,* 1999). Biological control was found to be an efficient tool for management of various plant diseases (Jacobsen and Backman) and our results are in accordance with their findings. In the past 10-15 years close to 4,000 publications have appeared in the field of plant growth-promoting bacteria (Bashan and Holguin 1998). Plant growth-promoting rhizobacteria enhance plant growth either by direct or indirect mechanisms (Glick 1995). The ways by which PGPR can influence plant growth directly may differ from species to species as well as from strain to strain.

The bacterial and fungal cultures were collected and maintained in appropriate medium. The plate and flask assay was performed for determining the antagonistic potential of *P.aeruginosa* and *P.fluorescens* towards *R.solani*. The plate assay demonstrates that the *R.solani* was not grown on plate with *P.aeruginosa* but grown well on plate with *P.fluorescens*. It indicates that *P. fluorescens* doesn't show any antagonistic activity towards *R. solani*. In the flask assay, the mycelia weight from the control was greater than that with *P.aeruginosa* (**Table. 1**). Dual bottom assay result also indicated that there is some volatile metabolites produced by *P. aeruginosa* which inhibits *R. solani*. Both plate and flask assay indicates that the *P.aeruginosa* shows antagonistic activity towards *R.solani*. Since *P.aeruginosa* has the inhibition potential, it was preceded further studies.

Plant growth promoting studies was performed by analyzing the various properties of *P.aeruginosa*. Autoinducing Metabolites was extracted from the *P. aeruginosa* and preserved in acetone. Poison plate method was performed by growing *R.solani* on different concentrations of AIM extract on PDA medium. From the result, it has been shown that 10 and 20 µg/ml concentrations shows moderate inhibition over *R.solani* whereas 40µg/ml shows complete inhibition of *R.solani*.

The production of HCN was determined by using method followed by Lorck, 1948. The colour of the filter paper was changed from yellow to brown which indicates the production of HCN. The siderophore production was also screened since it is an important coenzyme for most of the bacterial metabolic reactions. The yellow fluorescent pigment formed around the colony indicates the production of Siderophore.

IAA is one of the most important plant growth promoting hormones. IAA producing ability was analyzed by the method followed by Brick et al, 1991. Pink colour formation indicated the production of IAA. Phosphate is an important mineral which has to be solubilized for the plant uptake. The phosphate solubilizing ability was also screened using Pikovskaya agar medium (Pikovskaya 1948). Clear zones were observed around the colonies which confirm the phosphate solubilizing ability of *P. aeruginosa*.

The effect of co-culturing *P.aeruginosa* and *R.solani* was determined by growing them together and extracting metabolites from fungal mycelium. The extract from fungal mycelium grown with (test) and without (control) *P.aeruginosa* was runned on SDS-PAGE and stained by silver staining. The absence of few bands in test when compared with control indicates the inhibition of the production of few proteins of *R.solani* by *P.aeruginosa* due to co-culturing.

Quorum sensing was proved by co-inoculating the *R. solani* with bacterial cultures in

Table 1: Mycelial Dry weight of the *R. solani*

PDB	Pre weight	Dry weight
Test	1.222	3.131
Control	1.323	4.980

same plate. Same procedure was repeated by replacing *P. aeruginosa* with its AIM extract. Both shows significant inhibition of *R. solani*. It indicated that *P. fluorescens* also inhibited *R. solani* on co inoculation with *P. aeruginosa*. So, it has been proved that quorum sensing was happened between *P. aeruginosa* and *P. fluorescens* which stimulates the antagonistic activity of *P. fluorescens* towards *R. solani*.

The seed germination assay was carried out on seeds of Brinjal (*Solanum melongena*), Tomato (*Solanum lycopersicum*) and Ladys finger (*Abelmoschus esculentus)* (**Fig. 1**). Normal growth was observed in the control of all the three seeds.

The addition of *R. solani* completely inhibits the growth of tomato and Brinjal seeds and has no effect over ladys finger seeds. The addition of *P. aeruginosa* favours the average growth of all the

Control | *R. solani* | *R. solani* + *P. aeruginosa* | *R. solani* + *P.fluorescens* | *R. solani* + *P. aeruginosa* + *P.fluorescens*

Brinjal (*Solanum melongena*)

Tomato (*Solanum lycopersicum*)

Ladys finger (*Abelmoschus esculentus*)

Fig. 1: Germinated seeds by Seed germination assay

three seeds. Whereas *P. fluorscens* showed comparatively low plant growth promoting activity in the seeds when compared to *P. aeruginosa*. The addition of two strains ie *P. aeruginosa* and *P. fluorescens* shows good growth promoting activity towards tomato and brinjal seeds and shows average towards lady's finger.

CONCLUSION

The present study clearly demonstrated that, the *Pseudomonas aeruginosa* inhibits *R. solani* whereas *P. fluorescens* fails to inhibit *R. solani*. But when cultured together, both the strains of bacteria inhibit the growth of bacteria. It clearly indicates that the antagonistic activity of *P. fluorescens* was induced or stimulated by the *P.aeruginosa* when cultured together. *P.aeruginosa* was well known bacteria which involves in Quorum sensing in case of biofilm formation. The Seed germination assay was also shown that the combination of bacteria shows the increased germination rate of seeds. It serves as the strong evidence for the occurance of Quorum sensing between the *P. aeruginosa* and *P. fluorescens*.

P.aeruginosa was further analysed for the plant growth promoting activities like HCN production, Siderophore production, AIM, IAA and Phosphate solubilizing activity. *P. aeruginosa* shows positive for the entire test and hence shown to be having ability for being a biofertilizer. The specific effect of these Quorum sensing (QS) signals on the outcome of biological control can be further analyzed.

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