

Hydrogen Cyanide Mediated Biocontrol Potential of *Pseudomonas* sp. AMET1055 Isolated from The Rhizosphere of Coastal Sand Dune Vegetation

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Abstract

Twenty four hydrogen cyanide (HCN) producing fluorescent pseudomonads (FPs) were isolated from the rhizosphere of sand dune vegetation from Chennai coastal area. All the 24 cyanogenic (HCN producing) FPs were subjected to spectrophotometric assay to relatively quantify the amount of hydrogen cyanide (HCN) produced by them. Five FP strains designated as AMET1039, AMET1041, AMET1042, AMET1055 and AMET1064 produced more amount of HCN in their volatile fraction. In dual bottom plates assay, all these five isolates exhibited maximum mycelial growth inhibition of *Rhizoctonia solani* MML4001 due to the production of HCN. Among them, AMET1055 was found to be efficient and was identified as *Pseudomonas* sp. through various biochemical and staining techniques. The microscopical studies have concluded that the inhibitory activity of *Pseudomonas* sp. AMET1055 against *R. solani* MML4001 is of fungicidal nature. This study, reports for the first time that the rhizosphere of sand dune vegetation has cyanogenic FPs which can be used as biocontrol agents against soil borne fungal phytopathogens.

Keywords: Hydrogen Cyanide, Cyanogenesis, Fluorescent pseudomonas, Antagonism, Soil borne pathogens.

Introduction

Soil-borne pathogenic microorganisms affecting plant health are the main and constant menace to food production worldwide. *Rhizoctonia solani* Kuhn (teleomorph: *Thanatephorus cucumeris* (A.B. Frank) Donk) is a widespread and an ecologically diverse soil-borne fungus, causing different types of diseases in many plant species. It causes root rot, stem rot, fruit and seed decay, damping-off, foliar blight, stem canker and crown rot in various crops (Guleria *et al.*, 2007). Because of the flexible growth styles and wide spectrum of hosts, the management practices for the control of this most serious soil borne pathogen is always of chief interest. Over the past few decades, agricultural production has increased and farmers rely on chemical pesticides as a relatively dependable method of protecting plants against soil-borne pathogens. However, increasing use of chemical pesticides causes several negative effects on the environments as well as human health. Hence, biological control, the use of microorganisms for the control of plant diseases is gaining momentum since few decades (Mathivanan *et al.*, 2006).

Different mechanisms such as parasitism, production of volatile and non volatile antifungal compounds, competition for ferric iron, nutrients and colonization sites, and inducing systemic resistance in plants against pathogens are supposed to contribute to the biological suppression of soil-borne plant pathogens (Vasudevan *et al.*, 2002; Mathivanan *et al.*, 2006). Among these, production of volatile metabolites such as hydrogen cyanide (HCN) by soil bacterial biocontrol agents (BCAs)

against soil borne pathogens is most efficient but less studied mechanism in biological control of plant diseases.

Coastal sand dunes are common in different parts of the world. These are natural structures which protect the coastal environment by absorbing energy from wind, tide and wave action. Coastal sand dunes constitute a variety of microenvironments due to substrate mobility and physical processes. Plants establishing on coastal sand dunes are subjected to several environmental fluctuations such as high solar radiation, nutrient deficiency, drought, salt spray and high winds which affect their growth, survival and community structure (Oosting and Billings, 1942; Arun *et al.*, 1999). The microbial communities associated with these plants are also likely to be adapted to the different habitats available (Rosa *et al.*, 1995). However, exploration of coastal sand dune associated bacteria for beneficial activities are very scarce despite of their potential. In this paper, we are reporting the hydrogen cyanide mediated *in vitro* antagonistic activity of a rhizobacteria isolated from coastal sand dune vegetation.

Materials and Methods

Isolation of FPs

Ten gram of rhizosphere soil along with root bits of coastal sand dune plants were added to 95 ml of sterile distilled water in a conical flask. This suspension was serially diluted and spread plated on King's B agar

(KBA) medium (King *et al.*, 1954). The plates were incubated at room temperature ($30 \pm 2^\circ\text{C}$) for 48 h. The colonies that fluoresced under UV light were identified as FPs and distinct single colonies of FPs were picked, sub-cultured to purity on KBA (Jayaprakashvel *et al.*, 2007).

Detection of HCN production

Nutrient sucrose agar (NSA) medium was used to detect the production of HCN by the antagonistic bacteria as described by Lorck (1948). The production of HCN was determined by the change in color of the picric acid saturated filter paper from yellow to red-brown (Figure 1 & 2). Relative quantification of HCN was done following the spectrophotometric method described by Nagarajkumar *et al.* (2004).



Figure 1. Experimental set up for the detection of HCN production by bacteria



Figure 2. Color change of filter papers indicating the detection of HCN production

Effect of HCN on mycelial growth of *R. solani* MML4001: Dual bottom plates experiment

The plant pathogenic fungi *Rhizoctonia solani* MML4001 was obtained from Dr. N. Mathivanan, Biocontrol and Microbial Metabolites Lab, CAS in Botany, University of Madras, India. Selected cyanogenic FPs were made as lawn culture on NSA and each of its top lid was replaced with another bottom lid containing



Figure 3. Experimental set up for the dual bottom plates assay

freshly inoculated *R. solani* MML4001. This set up was sealed with parafilm and incubated for five days. Uninoculated NSA medium served as control (Fig. 3). The radial growth of *R. solani* MML4001 in all the treatments was measured at 2 and 5 days after incubation. The percent mycelial growth inhibition was calculated using the following formula.

$$\text{Inhibition of mycelial growth (\%)} = \frac{C-T}{C} \times 100$$

Here, C denotes the radial growth of *R. solani* MML4001 mycelium in control and T denotes the radial growth of *R. solani* MML4001 mycelium in cyanogenic bacterial treatments (Jayaprakashvel, 2008).

Identification of selected cyanogenic FP

The selected cyanogenic FP antagonist, AMET1055 was subjected to various biochemical and staining techniques as described by Cappuccino and Sherman (2004) and the results were interpreted with the key provided in the Bergy's Manual of Determinative Bacteriology (Holt *et al.*, 2004).

Microscopic Study

A microscopic study was carried out to find out the nature of antifungal activity of *Pseudomonas* sp. AMET1055 on *R. solani* MML4001. Mycelia from the edge of inhibitory point in dual plate were scraped gently, fixed and stained with lactophenol cotton blue. It was observed under a phase contrast microscope for mycelial abnormalities (Jayaprakashvel, 2008).

Statistical analysis

Data were analyzed for variance (one way ANOVA) using AGRES software. Per cent data were transferred in to arcsine values for which the statistical analysis was done.

Results and Discussion

Despite the worldwide use of synthetic chemicals such as fungicides and antibiotics to control the devastating effects of plant disease, the international agribusiness market still suffers extensive economic losses each year. Synthetic fungicides, pesticides, herbicides, and insecticides have not only failed to stop pests and pathogens, but have raised serious safety and environmental concerns. In this scenario, biological control of plant diseases offers natural alternatives to the fungicides and pesticides used in agriculture. A total of 32 FPs were isolated from the 13 soil samples collected from the rhizosphere of coastal sand dune vegetation at Chennai Coast. All the 32 FPs were found to exhibit fluorescence when they viewed under UV transilluminator. They were subcultured on KBA and the pure cultures were also tested for fluorescence. All the 34 FPs were tested for their ability to produce HCN. In the qualitative assay, 24 FPs have changed the color of sodium picrate impregnated filter paper strips, which clearly indicated that they are cyanogenic FPs (Table 1). However, the intensity of brown or reddish brown has varied between the FP isolates. All these 24 cyanogenic FPs were grown in NSB medium in test tubes for the quantification of HCN. This study has revealed that five isolates designated as AMET1039, AMET1041, AMET1042, AMET1055 and AMET1064 have produced higher amounts of HCN than the other isolates. Table 1 describes the amount of HCN produced by each of the cyanogenic FPs. Bacterial cyanogenesis appears to be essentially restricted to the proteobacteria *Chromobacterium violaceum* and to fluorescent pseudomonads (including many strains of *P. aeruginosa* and *P. fluorescens*, and some isolates of *P. aureofaciens* and *P. chlororaphis*) as well as certain cyanobacteria (*Anacystis nidulans*, *Nostoc muscorum* and *Plectonema boryanum*). Recently, some strains of *Rhizobium leguminosarum* have been reported to produce HCN as free-living bacteria (Castric 1981; Knowles and Bunch 1986).

Sl. No.	FP isolate	HCN Production (A625)
1	AMET1031	0.065 ± 0.005 ^{ef}
2	AMET1034	0.025 ± 0.002 ^{gh}
3	AMET1035	0.122 ± 0.007 ^c
4	AMET1036	0.078 ± 0.004 ^{de}
5	AMET1037	0.072 ± 0.001 ^{de}
6	AMET1038	0.082 ± 0.003 ^{de}
7	AMET1039	0.157 ± 0.001 ^b
8	AMET1041	0.155 ± 0.001 ^b
9	AMET1042	0.166 ± 0.002 ^{ab}
10	AMET1044	0.025 ± 0.000 ^{gh}
11	AMET1049	0.035 ± 0.003 ^g
12	AMET1050	0.043 ± 0.002 ^g
13	AMET1055	0.189 ± 0.001 ^a
14	AMET1056	0.072 ± 0.005 ^{de}
15	AMET1057	0.090 ± 0.005 ^{de}
16	AMET1058	0.076 ± 0.007 ^{de}
17	AMET1059	0.096 ± 0.004 ^{cd}
18	AMET1060	0.074 ± 0.003 ^{de}
19	AMET1061	0.080 ± 0.004 ^e
20	AMET1062	0.110 ± 0.003 ^c
21	AMET1063	0.100 ± 0.005 ^{cd}
22	AMET1064	0.158 ± 0.003 ^b
23	AMET1065	0.081 ± 0.006 ^{de}
24	AMET1071	0.085 ± 0.006 ^{de}

Table 1. Production of HCN by selected cyanogenic Fps

Values are mean of triplicates. Values in a column with same letters are not significantly different at 5% level.

An ecological role for bacterial cyanogenesis has been discovered for plant-beneficial strains of *P. fluorescens* and *P. putida*. The root-colonizing *P. fluorescens* strain CHA0 protects several plants from fungal root diseases (Voisard *et al.* 1994). As shown by mutant studies, HCN production accounts for a substantial part of the strain's biocontrol capacity, e.g. the suppression of tobacco black root rot caused by *Thielaviopsis basicola* (Voisard *et al.*, 1989). From the previous studies it is evident that cyanogenic FPs could be potential antagonists against many plant pathogens. Hence, the present study tested the antifungal activity of all the five efficient cyanogenic FPs in a specially designed dual bottom plates experiment. All the five isolates have inhibited the mycelial growth of *R. solani* MML4001 in different degrees (Table 2).

Sl. No.	Treatment	Mycelial growth inhibition (%) on different incubation periods	
		One day	Four days
1	Control	0.00 (1.65 ± 0.0) ^d	0.00 (1.65 ± 0.0) ^b
2	AMET1039	25.6 (30.4 ± 0.8) ^b	50.4 (45.2 ± 1.0) ^c
3	AMET1041	16.8 (24.1 ± 1.6) ^c	50.7 (45.4 ± 0.7) ^c
4	AMET1042	32.8 (34.9 ± 1.1) ^a	55.6 (48.2 ± 1.1) ^b
5	AMET1055	33.6 (35.4 ± 1.0) ^a	68.1 (55.7 ± 0.4) ^a
6	AMET1064	28.7 (32.4 ± 2.6) ^b	52.2 (46.3 ± 1.1) ^c

Table 2. Effect of cyanogenic FPs on the mycelial growth of *R. solani* MML4001

Values are mean of triplicates. Values in parentheses are the average of arcsine transformed values with standard deviation of per cent data for which the statistical analysis was done. Values in a column with same letters are not significantly different at 5% level.

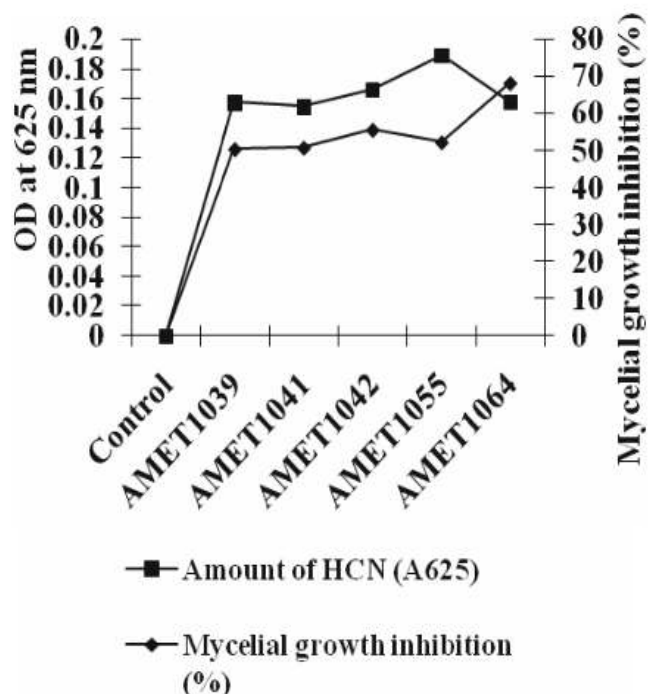


Figure 4. Comparative efficacy of HCN production and antifungal activity of cyanogenic FPs

The percent mycelia growth inhibition was recorded on two different days of incubation. On one day after incubation in dual bottom plates, all the isolates except AMET1042 and AMET1055 have exhibited lesser inhibition percentage. However, when they are observed on 4 days after incubation, all the isolates have produced more than 50% of mycelial growth inhibition. The mycelial growth inhibition against *R. solani* MML4001 by the selected five isolates was ranged between 52.2 - 68.1% (Fig. 4). AMET1055 has exhibited highest mycelia growth inhibition of 68% (Fig. 5) and hence it was then identified using various biochemical tests. The isolate was found to produce fluorescent pigments on KB medium. It is oxidase positive catalase positive and gram negative rod shaped bacterium with very good motility. It is not an endospore producer. All the above tests have convincingly indicated that the strain is *Pseudomonas* sp. Further, 16 s rRNA sequencing and other studies would help us to identify up to species level and the experiments are in progress.

Sl. No.	Biochemical test/ Staining method	Result
1	Gram staining	Negative
2	Endospore staining	Negative
3	Cell morphology	Short rods
4	Motility	Positive
5	Catalase	Positive
6	Oxidase	Positive
7	Fluorescence on KBA	Positive
8	Gelatin liquefaction	Positive

Table 3. Biochemical tests for the identification of cyanogenic FP strain AMET1055

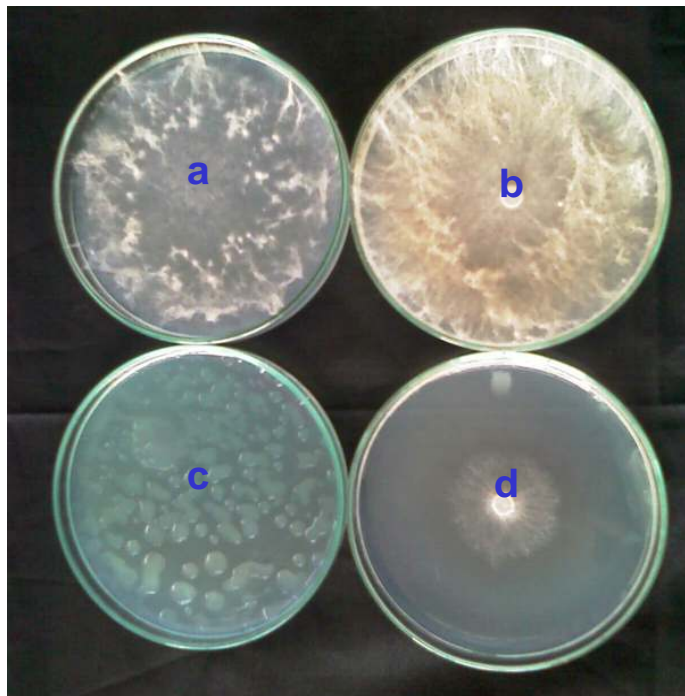


Figure 5. Effect of cyanogenic marine bacteria *Pseudomonas* sp. AMET1055 on the mycelial growth of *R. solani* MML4001

- Control NSA plate where *R. solani* MML4001 from the top PDA plate has overgrown
- Profuse growth of *R. solani* MML4001 in PDA plate
- Cyanogenic *Pseudomonas* sp. AMET1055 in NSA plate
- R. solani* MML4001 growth has been suppressed by the HCN produced by *Pseudomonas* sp. AMET1055

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