Biotec.Res.J.2015; Vol 1(2):139-145
eISSN 2395-6763

ORIGINAL RESEARCH

In vitro antifungal activity of *Bacillus spp* AV5 against pathogenic fungi *Colletotrichum falcatum*: Causal organism of Red Rot disease of Sugarcane

Rashid KAVIWALA[1], Mitesh DWIVEDI[1], Bishun Deo PRASAD[2], Prasant KUMAR[1]*
[1]C. G. Bhakta Institute of Biotechnology, Department of Fundamental and Applied Science,
UKA Tarsadia University, Bardoli, Surat-394 350, Gujarat, India
[2]Department of Plant Breeding & Genetics, Bihar Agricultural College, BAU, Sabour,
Bhagalpur-813210, Bihar, India
*Corresponding author email: prasant.kumar@utu.ac.in

• Received: 05 June 2015 • Revised: 06 July 2015 • Accepted: 11 July 2015 • Published: 28 July 2015 •

ABSTRACT
Red rot of sugarcane caused by *Colletotrichum falcatum* is the major devastating disease of sugarcane. This disease causes losses in both sugarcane yield and sugar recovery. Most commonly affected areas of India are Bihar, Punjab and Uttar Pradesh but now it spreads to coastal areas of Tamil Nadu and Gujarat. The primary aim of this study was to isolate *Bacillus* strains from different climatic condition and check the antifungal activity against three different strains of *Colletotrichum falcatum* isolated from affected sugarcane grown in South Gujarat. A total of 45 *Bacillus* strains were isolated from different samples. Seven out of these isolates showed significant inhibition against all the three strains of the pathogen *Colletotrichum falcatum* (i.e. CFNAV, CFTIM and CFKAM). One from these seven isolates, the *Bacillus spp.* strain AV5 showed the maximum inhibition (percentage) of the three fungus strains (i.e. 60, 58 and 55 respectively) suggesting its potential use as bio-control agent for the red rot of sugarcane.

KEY WORDS: *Antifungal, Bacillus, Collectotrichum, Sugarcane*

Introduction

Microorganisms have been shown to be attractive sources of natural compounds for pharmaceutical and agriculture industries. In agriculture, phytopathogenic fungi can cause plant diseases and much loss of crop yields. Pesticides are used to control plant pathogens. However, application of pesticides causes environmental pollution and decreased the diversity of non-target organisms. Microorganisms as biological control agents have high potential to control plant pathogens and cause no effect on the environment or other non-target organisms. There are numerous reports on the potential use of bio-control agents as replacements for agrochemicals (Shimizu *et al.,* 2000).

The genus *Bacillus* is one of the most common microorganisms used in the bio-control of phytopathogens. This genus comprehends a heterogeneous group of Gram-positive, aerobic or facultative anaerobic, endospore-forming bacteria. The endospores are thermotolerant structures, resistant to dryness, ultraviolet radiation and organic solvents. These properties, associated to the ability of producing peptide antibiotics, contribute to the utilization of this genus on the bio-control of several soil borne and foliar diseases (Backman *et al.,* 1997; Kloepper, 1997). Many *Bacillus* species are capable of producing a wide variety of secondary metabolites that are diverse in structure and function. The production of metabolites with antimicrobial activity is one of the factors to control plant pathogens (Silo-Suh *et al.,* 1994). These metabolites can be ribosomal

compounds such as subtilin (Zuber *et al.,* 1993), subtilosin A (Babasaki *et al.,* 1985), TasA (Stover *et al.,* 1999), and sublancin (Paik *et al.,* 1998). A variety of non-ribosomal produced small lipopeptides belonging to the surfactin family: iturin A, C, D, and E, bacillomycin D, F, and L, and mycosubtilin (Maget-Dana *et al.,* 1994); and the fengycin family: fengicins and plipastains (Vanittanakom *et al.,* 1986); as well as aminopolols such as zwittermycin A (Milner *et al.,* 1996) are also common. Antibiotics from the iturin family show strong antifungal and haemolytic activities with limited antibacterial activity shown by fengycin is specific against filamentous fungi and inhibits phospholopase A_2 (Muthamilan *et al.,* 1996; Nishikiori *et al.,* 1986). Surfactin shows antiviral and antimycoplasma activities (Vollenbrioch *et al.*, 1997a; Vollenbroich *et al.*, 1997b). Zwittermicin A is a linear aminopolyol (He *et al.,* 1994) having a broad spectrum of activity against certain Gram-positive, Gram-negative and eukaryotic microorganisms (Silo-Suh *et al,* 1998), and it also has an insecticidal activity similar to the protein toxin produced by *Bacillus thuringienesis* (Broderick *et al.,* 2000; Broderick *et al.,* 2003). *Bacillus spp.* especially *B. subtilis, B. cereus, B. licheniformis* and *B. amyloliquefaciens* have been effective against plant and fruit diseases caused by soil-borne, aerial, or post-harvest fungal pathogens (Broggini *et al*, 2005; Havenga *et al*, 1999; Korsten *et al*, 1995; Siddiqui *et al*, 1992; Szczech and Shoda, 2004; Yoshida *et al*, 2002). These bacteria produced lipopeptide surfactants and a diversity of polypeptide antibiotics with activity against bacteria and fungi. The current study deals with the screening of most potent *Bacillus spp* from different samples such as limestone, desert soil, garden soil and rhizospheric soil of medicinal plant *Adhatoda vasica* with the capability to inhibit fungal pathogen i.e. *Colletotrichum falcatum*; a causal organism of red rot disease of sugarcane.

Materials and Methods

Isolation of *Bacillus* spp. from soil samples

The samples were collected from limestone and desert soil of Rajasthan. The garden soil and rhizospheric soil sample of medicinal plant *Adhatoda vasica* were collected from Uka Tarsadia University campus, Gujarat. The soil samples (1 g) were suspended in normal saline solution (9 ml) and serially diluted to final dilutions of 10^{-4}, 10^{-5} and 10^{-6}. Aliquots (0.1 ml) from each dilution were spreaded on Hi chrome Bacillus Agar (Peptic digest of animal tissue 1%, Meat extract 0.1%, D-Mannitol 1%, Sodium chloride 1%, Chromogenic mixture 0.32%, Phenol red 0.0025%, Agar 1.5%; Final pH (at 25°C) 7.1±0.2, supplemented with 50 µg/ml cycloheximide to prevent growth of fungi, respectively; (Hi-Media Mumbai, India). Plates were incubated at 30°C for 1-2 days. Isolated Bacillus spp were further subcultured and maintained on Hichrome bacillus Agar. The isolates were preserved in glycerol solution and kept in deep freezer at -20°C.

Screening of *Bacillus* spp. for Antifungal activity against *Colletotrichum* spp

The dual culture method was used to screen the fungal growth inhibition capacity of *Bacillus spp. The Bacillus spp*. and *Colletotrichum spp* were inoculated at the opposite ends by using sterilized cork borer on the petriplates containing nutrient agar. Plates were incubated for two and three week, at 30 °C. Following the incubation, growth diameter of the pathogen was measured and compared with that of control (in which the bacterial suspension was replaced by sterile distilled water). The experiment was performed in triplicate using a single pathogen at a time. Three different *Colletotrichum falcatum* strains namely, CFNAV, CFTIM and CFKAM were obtained as a generous gift from Mr. Prittesh Patel, C. G. Bhakta Institute of Biotechnology, UKA Tarsadia University, Bardoli, Gujarat, India. The data was represented as percentage inhibition of colony growth using the Formula: (Growth of Control - growth of test)/Growth of Control (Percentage inhibition of colony growth) (Lokesha *et al.* 2007; Soytong, 1989).

Identification of bacteria

Isolated *Bacillus spp.* was grown in nutrient broth medium. The broth were harvested and centrifuged at 10,000 rpm for 2 min and washed twice with sterile distilled water. Further, the DNA was extracted by conventional NaCl-cTAB method (Sambrook and Russell, 2001). The 16S rDNA was amplified from the genomic DNA samples using universal primers 27F 5'-AGA GTT TGA TCC TGG CTC AG-3' and 1492R 5'- GGT TAC CTT GTT ACG ACT T-3'. The amplification was carried out in a 50 µL volume by using 20 ng of genomic DNA as template with 1X reaction Buffer (10 mM Tris pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$), 200 µM of each dNTP, 10 pM of each

primer and 0.05 U of Taq DNA polymerase (Merck, Banglore, India). The thermal cycling conditions were as follows: initial denaturation at 94°C for 5 min; 30 cycles at 95°C for 60 s, 54°C for 90 s and 72°C for 90 s; and a final extension at 72°C for 5 min. The amplification reaction was performed by thermal cycler (Eppendorf, Germany) and the amplified products were examined by 1% agarose gel electrophoresis. Phylogenetic tree was constructed by the neighbour-joining (NJ) method using the MEGA 6 software programme.

Results

Isolation of *Bacillus spp.* from limestone, desert soil, garden soil and rhizospheric of medicinal plant *Adhatoda vasica*

A total forty five isolates of *Bacillus spp* were obtained from four different sites *i.e.* 18, 10, 9 and 8 isolates were obtained from limestone, desert soil, garden soil and rhizospheric respectively.

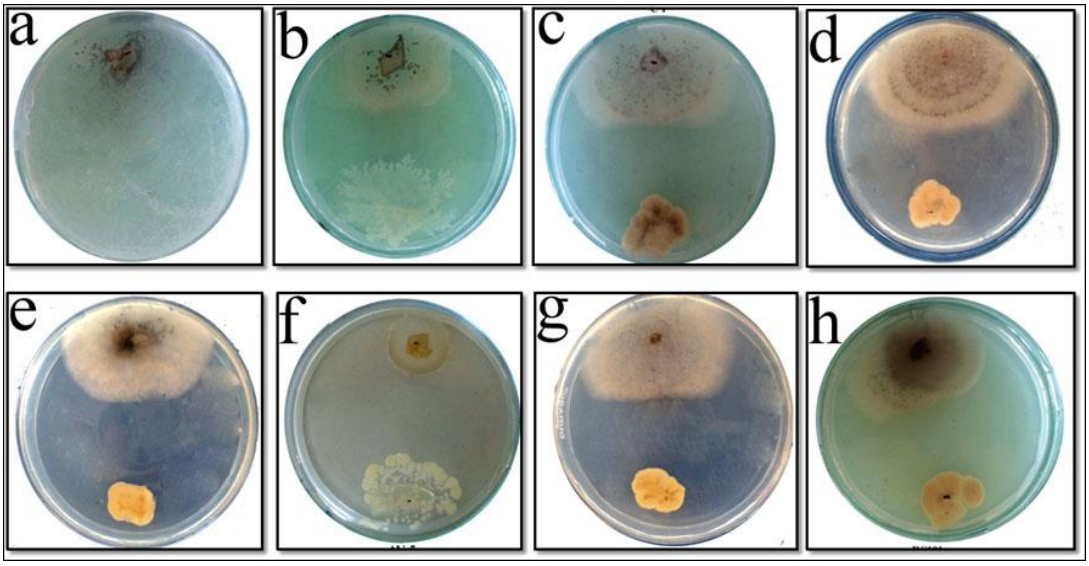

Figure 1: Antifungal activity against *Colletotrichum falcatum* strain CFNAV of the *Bacillus* bacteria isolated from limestone, desert soil, garden soil and Medicinal plant rhizosphere. (a) *Colletotrichum falcatum* strain CFNAV as a control; (b) strain ATG-2; (c) strain LS 11; (d) strain LS 3; (e) strain V5; (f) strain AV 5; (g) strain K52; (h) strain DS101 (Strain ATG-2; isolated from Garden soil; Strain V5 and AV5 isolated from Medicinal plant rhizosphere; Strain LS 3 and LS11 isolated from Limestone; Strain K52 and DS101 isolated from Desert Soil.

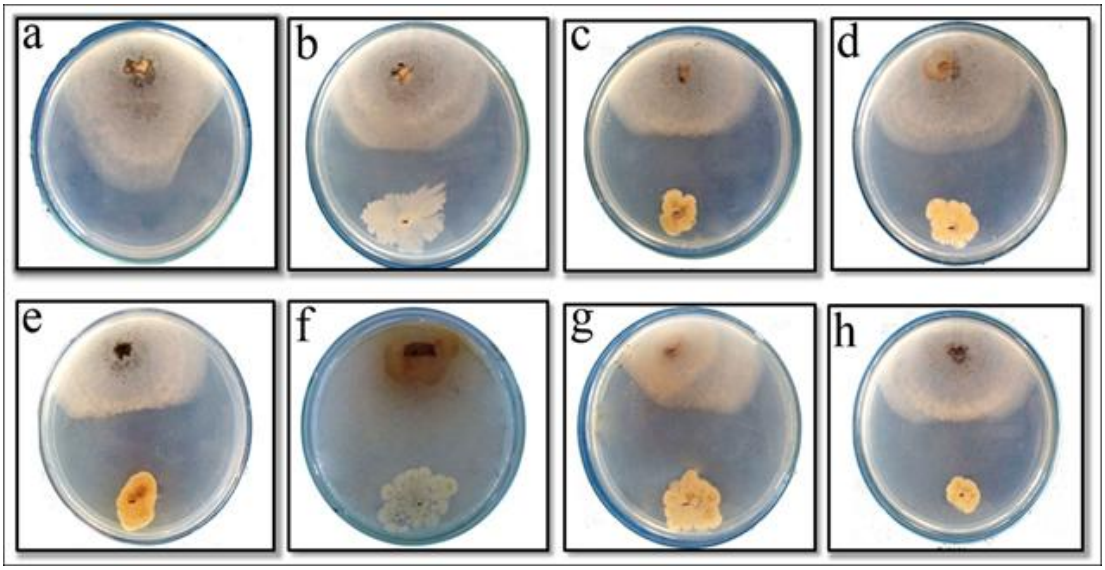

Figure 2: Antifungal activity against *Colletotrichum falcatum* strain CFTIM of the *Bacillus* bacteria isolated from limestone, desert soil and Medicinal plant rhizosphere. (a) *Colletotrichum falcatum* strain CFTIM as a control; (b) strain ATG-2; (c) strain LS 11; (d) strain LS 3; (e) strain V5; (f) strain AV 5; (g) strain K52; (h) strain DS 101

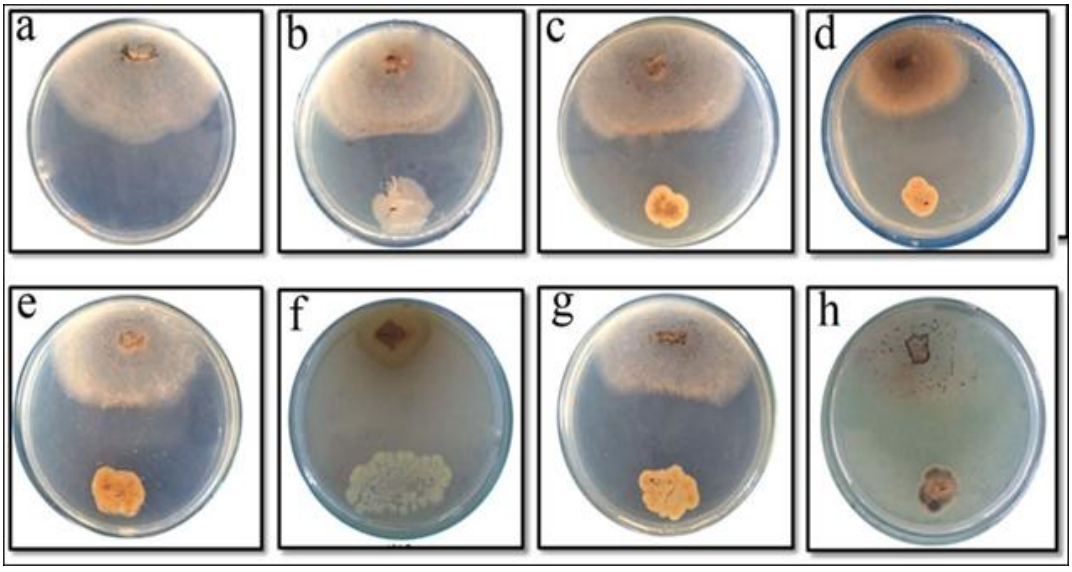

Figure 3: Antifungal activity against *Colletotrichum falcatum* strain CFKAM of the *Bacillus* bacteria isolated from limestone, desert soil and Medicinal plant rhizosphere. (a) *Colletotrichum falcatum* strain CFKAM as a control; (b) strain ATG-2; (c) strain LS 11; (d) strain LS 3; (e) strain V5; (f) strain AV 5; (g) strain K52; (h) strain DS 101

Screening of *Bacillus spp* for antifungal activity against *Colletotrichum falcatum*

Screening was performed on the basis of dual culture plate inhibition method. A total of 7 of 45 isolates of *Bacillus spp* produced zone of inhibition against all the three strains of *Colletotrichum falcatum* (Figure 1, 2 and 3). Further, the percent inhibitions against *Colletotrichum falcatum* were calculated for all the seven *Bacillus* isolates and the inhibition experiments were performed in triplicates for all the three *Colletotrichum falcatum* strains (i.e. CFNAV, CFTIM and CFKAM) (Table. 1).

Identification of most potent antifungal producing *Bacillus spp*

The most potent antifungal producing *Bacillus spp* AV5 was identified by amplification and sequencing of the 16S rRNA. The 16S rRNA gene sequence of *Bacillus spp* AV5 was submitted to GenBank (Accession number: KP137512). The information obtained by the BLAST program revealed that the isolate selected by on Hichrome bacillus agar and gram staining procedures were matched with *Bacillus* spp. Further, the phylogenetic analysis confirmed the identity (Figure 4).

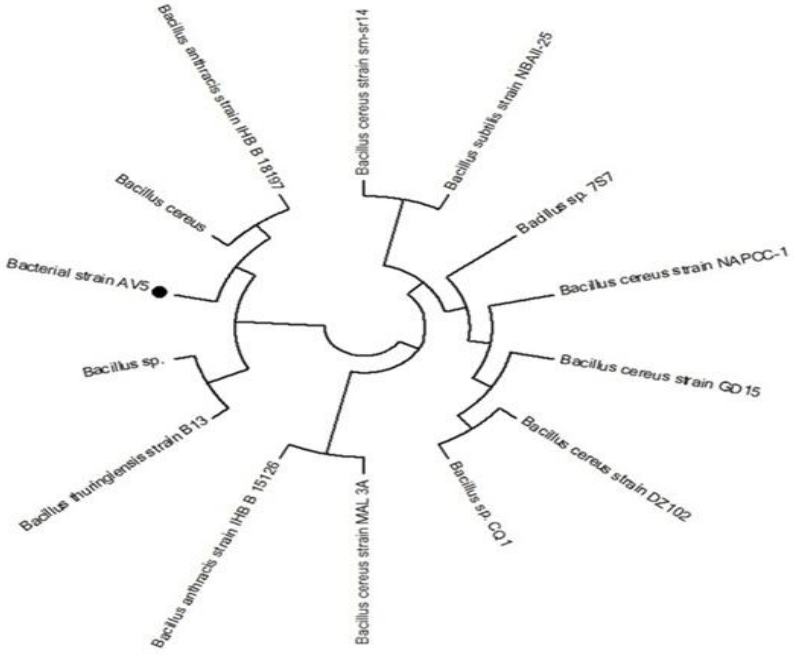

Figure 4: Phylogenetic tree of the Gram positive *Bacillus spp*. strain AV-5.

Table 1. Antifungal activity of *Bacillus* strains against *Colletotrichum falcatum*

	2 Weeks				3 Weeks		
Colletotrichum falcatum	***Bacillus spp.***	**Mean ± SEM**		**Inhibition(%)**	**Mean ± SEM**		**Inhibition (%)**
CFNAV	Control	4.00 ± 0.003	4	NA	4.20 ± 0.003	4.2	NA
	K52	2.36 ± 0.067	2.4	41.00	2.60 ± 0.058	2.6	38.1
	DS101	2.63 ± 0.067	2.6	34.25	2.65 ± 0.150	2.65	36.9
	LS11	2.47 ± 0.120	2.5	38.50	2.50 ± 0.100	2.5	40.5
	ATG2	2.47 ± 0.033	2.5	38.50	2.50 ± 0.050	2.5	40.5
	V5	2.67 ± 0.167	2.7	33.50	2.85 ± 0.250	2.85	32.1
	LS3	2.83 ± 0.167	2.8	29.25	3.05 ± 0.450	3.05	27.3
	AV5	1.60 ± 0.001	1.6	60	1.79 ± 0.135	1.79	58.0
CFTIM	Control	4.6 ± 0.100	4.6	NA	5.7 ± 0.800	5.7	NA
	K52	2.23 ± 0.030	2.2	51.52	2.50 ± 0.022	2.5	56.1
	DS101	2.43 ± 0.145	2.4	47.17	3.10 ± 0.033	3.1	45.6
	LS11	2.70 ± 0.153	2.7	41.30	2.60 ± 0.200	2.6	54.4
	ATG2	2.83 ± 0.203	2.8	38.47	3.40 ± 0.033	3.4	40.3
	V5	2.56 ± 0.067	2.6	44.34	2.70 ± 0.100	2.7	52.6
	LS3	3.33 ± 0.067	3.3	27.60	3.20 ± 0.300	3.2	43.8
	AV5	1.90 ± 0.067	1.9	58.71	2.30 ± 0.033	2.3	59.3
CFKAM	Control	3.6 ± 0.100	3.6	NA	4.55 ± 0.650	4.5	NA
	K52	2.4 ± 0.115	2.4	33.3	2.60 ± 0.088	2.60	42.2
	DS101	3.33 ± 0.167	3.3	7.5	3.25 ± 0.250	3.25	27.7
	LS11	2.67 ± 0.167	2.6	25.83	2.73 ± 0.145	2.73	39.3
	ATG2	3.00 ± 0.170	3.0	16.6	3.00 ± 0.033	3.00	33.3
	V5	2.70 ± 0.208	2.7	25	2.65 ± 0.250	2.65	41.1
	LS3	3.07 ± 0.088	3.0	15	3.00 ± 0.252	3	33.3
	AV5	1.60 ± 0.033	1.6	55	2.3 ± 0.220	2.3	48.9

(Zone of inhibition measured in cm and values were calculated as percentage inhibition and expressed as Mean ± SEM; NA refers to not applicable).

Discussion

India ranked fifth in the world for growing sugarcane on area basis whereas the position is twelfth in the recovery of sugar from the cane (Kumar *et al.,* 2014). Red rot of sugarcane caused by *Colletotrichum falcatum* is the major devastating disease of sugarcane. As far as India is concerned this disease is present in subtropical India especially Bihar, Punjab and Uttar Pradesh but now it spreads to coastal Tamil Nadu and Gujarat as well. The yield loss due to red rot may go up to 100 percent. Development of new variants of the fungus and environmental pollution concerned with the excessive use of agro-chemicals have resulted in adopting the biological control using native strains of plant associated rhizobacteria as a supplemental strategy to minimize pesticides usage (Muthamilan and Jayarajan,1996). Inclination towards the use of biological organism to control the pest and diseases are mainly due to environmental pollution and toxicity of pesticides. In this study, we isolated 45 *Bacillus spp* and subsequently checked the inhibitory effects on the growth of three *Colletotrichum falcatum* isolated from different sugarcane varieties grown in the regions of Gujarat. Seven out of 45 isolates showed significant inhibition against all the three species of the pathogen i.e. CFNAV, CFTIM and CFKAM. Of these seven

isolates, the maximum inhibition of the fungal species (i.e. 60, 58 and 55 against the three strains of the pathogenic strains respectively) was shown by the AV5 (GenBank Accession number: KP137512). Thus, our finding suggest that the *Bacillus spp* AV5 isolated from the rhizosphere of medicinal plant *Adhatoda vasica* had a better inhibitory activity against the selected *Colletotrichum falcatum* strains as compared to all other isolates obtained from different soil samples and geographical areas. Moreover, this preliminary study suggests that rhizospheric microflora of medicinal plants could be a better choice for isolating the potent antifungal organisms for important phytopathogens.

Conclusions

The presented data exhibit the antifungal activity of *Bacillus spp* AV5 and indicate the possibility of using the *Bacillus spp* AV5 as a biological control agent against red rot of sugarcane disease caused by *Colletotrichum falcatum*. However, field evaluation and bioactive compounds identification are necessary to determine its efficacy under natural ecosystem.

Acknowledgements

We are grateful to the C. G. Bhakta Institute of Biotechnology, Uka Tarsadia University, Surat, India to provide financial support and laboratory facilities to carry out the present investigation.

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