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MORPHOLOGICAL AND BIOCHEMICAL CHARACTERIZATION OF *Xanthomonas campestris* (PAMMEL) DAWSON PV. *SESAMI* AND IT'S MANAGEMENT BY BACTERIAL ANTAGONISTS

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Isolates of *Xanthomonas campestris* (Pammel) Dawson pv. *sesami* (Xcs) were collected from various locations in Pakistan and were characterized using several biochemical tests including; (KOH) string test, gram staining, H_2S production, catalase activity, oxidation/fermentation of glucose, oxidase activity and nitrate reduction. The isolates were positive in tests for KOH string assay, H_2S, catalase and showed negative reactions in gram staining, oxidase test and nitrate reduction while oxidative utilization of glucose was observed. To develop biological control for the pathogen we isolated numerous bacteria from the rhizosphere of sesame plants and screened them for antagonism against the pathogen using the zone inhibition method. Out of 87 isolates, 9 isolates were inhibitory to the pathogen *in vitro*. *Pseudomonas fluorescens* ID-3 exhibited the highest zone of inhibition i.e. 7.97mm. All antagonistic isolates were tested for disease control under greenhouse conditions. Four sesame lines that were found to be moderately resistant in previous studies (i.e. 95001, 96007, 96019, and 20003) were selected for evaluating biocontrol efficacy. The isolates were found to control the disease and reduced severity to 3% compared to untreated plants. The isolate *P. fluorescens* ID-3 was considered to provide the best disease control with each cultivar. Average biocontrol efficiency (BE) of ID-3 was 78.25% while the best BE was obtained in line 96019, i.e. 81%.

Keywords: sesame, *Xanthomonas campestris* pv. *sesami*, biochemical, morphology, biomanagement, rhizobacteria, *Pseudomonas fluorescens.*

INTRODUCTION

Sesame is chiefly produced in tropical and sub-tropical regions (Ashri, 1998), on 6.5×10^6 ha of area worldwide yielding more than 3×10^6 of seed (FAO, 2005). Globally Pakistan is the 14th largest producer of sesame. It is the most common crop grown in the Potohar region where it is locally called *Til*. Sesame originated in East Africa and India and it has been an integral part of food consumption and ancient legends (Bedigian, 1985; Nayar and Mehra, 1970).

Diseases, along with other factors, are important constraints in the production of sesame. Bacterial diseases of importance include leaf spot and bacterial blight. Leaf spot caused by *P. syringae* pv. *sesami* can reduce yields, but bacterial blight caused by the *X. campestris* pv. *sesami* (*Xcs*) is far more damaging during the monsoon season (Maiti *et al.*, 1985).

The sesame seed comprises 50-60% oil and up to 40% protein. The average of linoleic and oleic acid accounts for 83% of unsaturated fatty acids which enhances the appropriateness of sesame for consumption by humans. Bacterial blight was first discovered in Pakistan in 1986 (Akhtar, 1986). The disease is most damaging when continuous humid weather conditions prevail. Survey data of 30 fields in Punjab (Pakistan) confirmed that sesame bacterial blight is widely distributed and is a serious disease requiring immediate attention to alleviate its severity (Ahmed, 2004).

This disease is now considered as a threat to the industry in Pakistan (Ahmed, 2004). Numerous strains or pathovars of *Xcs* occurring are found over a wide range of ecological conditions and these cannot be differentiated precisely (Sutic and Dawson, 1962).

Many bacteria growing in the rhizosphere that increase plant growth by various mechanisms have been termed as plant growth promoting rhizobacteria (PGPR). These mechanisms can involve growth stimulation that impacts the plants' physiology directly via substances which promote growth or increase nutrient availability in soil, or through indirect activities such as suppression of plant pathogens in the rhizosphere (Ahmad *et al.*, 2008).

It has been shown from the previous studies that there is no variety/line of sesame resistant to bacterial blight (Naqvi *et al.*, 2012). The aims of this study are to characterize the *Xcs*

isolates present in Pakistan for pathogenicity and characterize them using biochemical tests. Currently there is no information regarding the biological suppression of *Xcs*. Therefore we examined the potential for biocontrol by making a collection of rhizobacteria that can antagonize the pathogen. This study would be a step forward toward the integrated disease management of bacterial diseases of sesame.

MATERIALS AND METHODS

***Isolation of X. campestris pv. sesami*:** The lesions and surrounding healthy tissues were cut into small pieces and washed in tap water before surface sterilizing in 1 % sodium hypochlorite for 2-3 minutes. Following the pieces were immersed in 70% ethanol. Disinfected pieces were rinsed in sterilized distilled water and dried on sterile blotting paper. These pieces were placed on solidified NA medium (Hung and Annapurna, 2004). Seeds were also surface sterilized and grinded in small quantity of water. The suspension was placed on NA medium. Petri dishes were incubated at 28±2 °C and observed for bacterial growth 4-6 days later. The bacterial cultures were purified by streaking until pure cultures were obtained.

***Hypersensitivity*:** Isolated colonies of *Xcs* were tested for ability to induce a hypersensitive response on foliage of the non-host plant tobacco (*Nicotiana tabacum* cv. *burley*). Test isolates were freshly grown on NA at 30±2°C for 24 h and suspended in sterile water, maintaining an inoculum concentration of 10^8 cfu/ml (using Genie Spectrophotometer at 550 nm). Tobacco seedlings were inoculated with suspensions of the selected isolates at the 5-6 leaf stage. 1 ml of bacterial suspension was injected using a disposable syringe into the lower surface of the tobacco leaves and the plants were sealed in plastic bags for 24 h to prevent desiccation. Plants were uncovered and kept in the laboratory at 28±2°C for 36 h. Control plants were injected with sterilized distilled water and kept under same conditions (Klement *et al.*, 1964).

***Virulence test*:** For the assessment of pathogenicity of *Xcs* on sesame, an inoculum suspension was prepared in sterile water. The methods of inoculation used were: pin prick, sand paper and injection. In the pin prick method, paper pins were used to bruise the seeds and the inoculum (10^6 cfu/ml) of *Xcs* was applied. In sand paper method, abrasions were made on seeds using sand paper and the inoculum was applied on the injured surfaces. In the third method, injection, leaves of young seedlings were inoculated with 1 ml of inoculum using a hypodermic syringe (as described above). Control leaves were inoculated with sterilized distilled water in all cases.

***Characterization of* Xcs *isolates*:** The temperature effect on the growth and colony features of the isolates on different media (NA, YDCA and YDA) were determined. The isolates were subjected to several biochemical tests that included KOH string test, gram staining, H_2S production, catalase test, oxidation/fermentation of glucose, oxidase test, and nitrate reduction assay (Schaad *et al.*, 2001).

***Isolation of rhizobacteria*:** Rhizobacterial isolates were isolated on Nutrient Agar (NA) medium which was prepared, autoclaved and poured in Petri plates of 7.6 cm diameter. For isolation, 10 g of rhizospheric soil was collected and suspended in 100 ml PBS and mixed thoroughly. Serial dilutions were made and 1 ml of each of the dilutions of 10^{-5} – 10^{-7}, were spread on media. Rhizobacteria were isolated and purified by streaking until the pure cultures were obtained. The Petri plates were incubated at 30±2ºC.

***In vitro antagonistic activity of rhizobacteria*:** The antagonist activity of isolates against *Xcs* was tested according to the method of Mitchell and Carter (2000). The 100µl suspension of *Xcs* containing 10^8 cfu ml^{-1} was spread on KB/LB agar medium and four holes of 9 mm diam. were punched into the agar. Into these holes 30µl supernatant, obtained after centrifugation and filtration by 0.22µm filter (Millipore) of a 48-h-grown culture of each test antagonist (10^9 cfu ml^{-1}) was added and the plates were incubated at 30 ^oC for 48 h. Bacterial isolates were selected on the basis of inhibition zone against *Xc*s.

***Greenhouse evaluation of antagonists*:** Four sesame lines (95001, 96007, 96019 and 20003) that proved moderately resistant to bacterial blight in previous studies were used (Naqvi *et al.*, 2012). Seeds were surface sterilized using 1% mercuric chloride and washed several times with distilled water to remove the excessive mercuric chloride. The growth medium (soil 1: sand 1: compost 1) was also sterilized by autoclaving and pots were filled. The rhizobacteria that proved antagonistic during *in vitro* tests were selected for green house evaluation. Rhizobacterial inoculum was prepared in LB media by shaking for 72 h and the concentration was adjusted to 10^9 cfu ml^{-1} for each isolate. Seeds were soaked in the inoculum for 30 min and dried before sowing. Pathogen inoculum was prepared by the same method and the suspension was adjusted to 10^8 cfu ml^{-1}. Ten ml of the inoculum was added to each pot. Data of disease incidence was collected after 50 days on the basis of disease rating scale (Sarwar and Haq, 2006).

Biocontrol efficiency (BE) of the isolates was recorded (Guo *et al.*, 2004):

BE (%) = ([disease incidence of control - disease incidence of treatment group]/disease incidence of control) x100.

***Identification of Rhizobacteria*:** The antagonistic isolates were identified using Bergey's manual of determinative bacteriology (Holt *et al.*, 1994), Seldin and Penido (1986), Claus and Berkeley (1986) and Gordon *et al.* (1973).

Data Analysis: The data was analyzed by analysis of variance (ANOVA) at 5% level of significance using SPSS version 17.0.

RESULTS

***Isolation of Xcs*:** Sixteen isolates were obtained from samples collected from various locations. These isolates differed in morphological appearance i.e. color, shape etc. In general the colonies were shiny yellow which is characteristic of *Xanthomonas* spp.

***Hypersensitivity test*:** Eleven of 16 isolates tested on tobacco plants induced a hypersensitive reaction within 24 – 58 h after inoculation and based on these reactions, were categorized as strongly, moderately, or mildly virulent. The isolates that failed to cause a reaction within 72 hours after inoculation were considered to be non-pathogenic. The isolates NARC-1, 4 and 7 and Gojra-4 and 8 were considered as highly virulent, whereas NARC-2, 3, 6 and 8 were moderately virulent. The isolates Gojra-1 and 5 were regarded as weakly virulent and NARC-5, Gojra-2, 3, 6 and 7 were considered to be non-pathogenic. No symptoms were recorded in controls inoculated with distilled water (Table 1).

Isolates identified as non-pathogenic were re-cultured on YDCA and again tested for the hypersensitive reaction on tobacco, with the same results. These non-pathogenic isolates were not used in further biochemical tests.

Table 1. Hypersensitive responses of the different isolates tested on tobacco

Isolates	Hypersensitive reaction (+/-)	Duration (hrs)	Virulence
NARC-1	+	24	High
NARC-2	+	36	Moderate
NARC-3	+	26	Moderate
NARC-4	+	24	High
NARC-5	-	72	Non-pathogenic
NARC-6	+	36	Moderate
NARC-7	+	24	High
NARC-8	+	36	Moderate
Gojra-1	+	58	Weak
Gojra-2	-	72	Non-pathogenic
Gojra-3	-	72	Non-pathogenic
Gojra-4	+	24	High
Gojra-5	+	58	Weak
Gojra-6	-	72	Non-pathogenic
Gojra-7	-	72	Non-pathogenic
Gojra-8	+	24	High
Control	-	24-72	---

***Virulence test*:** All the isolates that induced a hypersensitive response on tobacco leaves were tested for virulence on sesame plants. All inoculation methods used (pin prick, injection and sand paper) gave positive responses on the host plant.

All isolates tested were ranked as highly, moderately, or weakly virulent pathogens, based on the host response over the 7 days of observation. NARC-1 and 6 and Gojra-5 and 8 were highly virulent. Amongst these highly virulent isolates, symptoms in plants inoculated with NARC-1 developed earlier than with other isolates, in 6 days, whereas with other isolates symptom development was slower. NARC-6 induced less symptom development than NARC-1 in the first six days after inoculation, but in the following six days symptoms developed at a faster rate. No symptoms developed in controls treated with sterile water in the 15 days of observations (Table 2).

Table 2. Virulence of *Xcs* isolates on sesame, with ranking

Isolates	Observations			Reaction			**
	1st	2nd	3rd	PPM	SPM	IM	Ranking
NARC-1	+++	+++	+++	✓	✓	✓	SP
NARC-2	++	++	++	✓	✓	✓	MP
NARC-3	+	++	++	✓	v	✓	MP
NARC-4	++	+++	+++	✓	✓	✓	MP
NARC-6	++	++	++	✓	✓	✓	SP
NARC-7	++	++	++	✓	✓	✓	MP
NARC-8	+	++	++	✓	v	✓	MP
GOJRA-1	+	+	++	✓	✓	✓	MP
GOJRA-4	+	+	++	✓	v	✓	WP
GOJRA-5	++	+++	+++	✓	✓	✓	SP
GOJRA-8	++	+++	+++	✓	✓	✓	SP
CONTROL	-	-	-	NO REACTION			--

PPM: pin prick method; SPM: sand paper method; IM: injection method; SP: strong pathogenic; MP: moderately pathogenic; WP: weak pathogenic; NP: non-pathogenic

***Characterization of Xcs isolates*:** It was observed that the colonies were yellowish, smooth and shiny. The best growth was observed on YDCA and NA at 25-27ºC and 26ºC respectively. These were yellow on YDA at 28ºC, smooth and shiny yellow on YDCA at 27-30ºC.

Within the NARC isolates differences in colonial morphology was found when the isolates were cultured on different media. NARC-1 and 2 produced shiny yellow colonies on NA, while isolate 3 and 5 produced yellowish colonies. However, on YDA all the isolates produced yellow colonies. Almost the same characteristics were found in colony morphologies with the Gojra isolates with shiny yellow colonies on NA, yellowish on YDA, smooth and shiny yellow on YDCA.

The best temperatures for growth on NA, YDA and YDCA media were 26, 28 and 29ºC. However, the temperature ranged between 25-29°C, 27-29°C and 29-30°C for NA, YDA and YDCA respectively indicating that the isolates may have genetic differences.

KOH-*String test*: All isolates responded positively to loop test by forming a thread when uplifted gently. The loop

formation is confirmation of gram negative bacteria and the above mentioned isolates were Gram negative (Halebian *et al*., 1981; Suslow *et al*., 1982). Each isolate was tested twice and same results were revealed.

***Gram Staining*:** The isolates marked as Gram negative from the loop test were tested using Gram staining. All isolates retained a pinkish color thus confirming that they were gram negative. The test was executed twice and similar results were obtained.

For the observation of cell size and shape, the counterstained slides were observed using a microscope at 600X magnification. Each slide, representing one isolate, was examined and the shape of the bacteria found to be elongated rods with an average size of 1.04 µm x 0.5 µm (L x W). NARC isolates sizes however, ranged between 0.7-1.8 µm x 0.4-0.8 µm whereas, the Gojra isolates ranged between 0.6-1.6 µm x 0.3-0.8 µm.

***H_2S production*:** All isolates tested positive for H_2S production giving a black discoloration on lead acetate paper strips.

***Catalase test*:** All the isolates of *Xcs* were found catalase positive and gave off H_2O_2 bubbles in the test petri plates.

***Oxidation/fermentation of glucose*:** All isolates showed a yellow color change in test tubes without mineral oil indicating the capacity to oxidize glucose.

***Oxidase test*:** All the test isolates were oxidase negative and did not show purple color even after 60 sec.

***Nitrate reduction*:** All isolates were unable to reduce nitrate to nitrite even after the addition of zinc.

***In vitro antagonistic activity of rhizobacteria*:** Out of 87 rhizobacterial isolates, only 9 isolates proved antagonistic during the *in vitro* studies. The isolate ID-3 produced the largest zone of inhibition (7.97 mm) against *Xcs* followed by ID-12 which showed 6.5 mm zone of inhibition (Table 3).

Table 3. Assessment of antagonism of rhizobacterial isolates by zone of inhibition

Isolates	Zone of inhibition±S.D* (mm)
FD-9	4.50 ± 0.30 c
FD-17	3.77 ± 0.31 d
FD-21	3.23 ± 0.15 e
ID-3	7.97 ± 0.21 a
ID-12	6.43 ± 0.12 b
TTS-5	3.10 ± 0.60 e
TTS-7	4.67 ± 0.15 c
GJ-1	1.90 ± 0.10 f
GJ-4	3.97 ± 0.42 d
Control	0.00 ± 0.00 g

*S.D= Standard deviation

***Biological Control of bacterial blight of sesame under greenhouse*:** Tests conducted in the greenhouse with the antagonists for control of bacterial blight disease showed that all the isolates suppressed the disease but to a different extent. Isolate ID-3 was the most effective with average BE of 78.25%. The best BE was obtained with line 96019 of 81%. ID-12 provided an average BE 62.25%. Line 20003 had the least incidence of disease as observed with the inoculated control and as compared to other lines with the same treatment (Table 4).

***Identification of rhizobacteria*:** All the isolates were identified; isolates FD-9, FD-17, ID-3, TTS-7 and GJ-1 belonged to *Pseudomonas fluorescens* and isolates FD-21, ID-12 and GJ-4 were identified as *Bacillus subtilis* and TTS-5 as *Paenibacillus polymyxa*.

DISCUSSION

The results of the different biochemical tests carried out on the pathogenic isolates indicated that the isolates are likely *Xanthomonas* spp. (Abdul-Rahim and Adam, 1990). The fact that they were Gram negative rod shaped bacteria as

Table 4. The effect of rhizobacteria isolates on the bacterial blight incidence under greenhouse conditions

Isolates/Lines	95001		96007		96019		20003	
	DI (%)	BE (%)	DI (%)	BE (%)	DI (%)	BE (%)	DI (%)	BE (%)
FD-9	9 a	51 bc	10 def	48 bcd	8 de	49 d	6 defg	55 abc
FD-17	11 cd	40 cd	11 cde	43 cd	9 cd	35 cde	7 cdef	43 bcd
FD-21	12 c	34 d	13 c	33 de	10 c	32 de	8 bcd	33 bcde
ID-3	4 f	77 a	5 g	77 a	3 g	81 a	3 g	78 a
ID-12	6 ef	64 ab	8 f	60 ab	5 f	63 ab	4 fg	62 ab
TTS-5	12 c	32 d	11 cde	41 cd	11 bc	22 e	8 bcd	28 cdef
TTS-7	8 e	55 bc	9 ef	53 bc	7 ef	55 bc	5 efg	62 ab
GJ-1	16 b	14 e	16 b	17 e	12 b	16 ef	10 ab	11 ef
GJ-4	11 cd	43 cd	12 cd	37 cd	11 bc	24 e	9 abc	23 def
Control	18 a	0 e	19 a	0 f	15 a	0 f	12 a	0 f

DI= Disease Incidence, BE= Biocontrol efficiency

Figures with different alphabets are significantly different from each other

observed by Kottle (1985). The size, growth characteristics on various media and yellow, smooth and shiny colonies also supported the isolates as Xanthomonas species (Dye, 1962; Kado and Heskett, 1970; Schaad and White, 1974; Kottle, 1985). The genus *Xanthomonas* was separated from other plant pathogenic bacteria on the basis of the motile, yellow coloured cells, with a single polar flagellum, mostly producing acids in media containing lactose. In naming the genus, the yellow colour of the bacteria, which is one of the most significant characteristics of the genus (Sabet and Dowson, 1960). Since then the yellow color has been an invaluable aid in the identification of *Xanthomonas* (Nancy *et al*., 1988).

The isolates showed differences in cell sizes indicating some variability among isolates collected from different localities (Ahmed, 2004). Different sizes of the bacteria were observed that may be due to the difference in age of the bacteria.

Only *P. fluorescens* ID-3 provided significant protection to plants from the pathogen. The line 96019 which had been categorized as moderately resistant (DI=10.1-20%) according to the scale of Sarwar and Haq (2006) in a previous study by Naqvi *et al.* (2012) could now be considered as highly resistant after inoculation with antagonistic rhizobacteria as disease incidence was reduced to 0.1-5%. Reduction of disease may be due to the enhanced lignin content that strengthens the walls, increase in peroxidase activity and 4-coumarate CoA ligase activity was reported by Vidhyasekaran *et al.* (2001). Such biochemical changes were also observed after inoculation with *Xanthomonas oryzae* pv. *oryzae* in rice leaves pre-treated with *P. fluorescens.* Several workers have reported *P. fluorescens* as effective biocontrol agent against several pathogens (Buysens *et al*., 1996; Singh *et al*., 2006). *P. fluorescens* could stimulate and enhance plant defense reactions (M' Piga *et al*., 1997). Recent studies imply that prior application of fluorescent pseudomonads strengthens host cell wall structure that results in restriction of pathogen invasion in plant tissue (Benhamou *et al*., 2000; Chen *et al*., 2000; Conrath *et al*., 2002; Dwivedi and Johri, 2003). The role of secondary metabolites like antibiotics, siderophores also seems to be governing the suppression of pathogens (Meziane *et al*., 2005). In the current study highest zone of inhibition was observed by *P. fluorescens* isolate ID3 implying that it might be producing strong antimicrobial compounds.

Both *Bacillus* and *Paenibacillus* species are well known for the antagonizing ability against the pathogens under *in vitro* and *in vivo* conditions (Arrebola *et al*. 2010; Chen *et al*. 2009). Plant growth promotion by inhabiting the roots of the plants by both these bacteria is well documented. These utilize root exudates of plants and in turn provide them with unavailable nutrients as well as defend the plants against pathogens (Kloepper *et al*., 2004). Our study also found both these bacteria as potential antagonists which can be used as biocontrol agents against bacterial pathogens as well. The *B. subtilis* isolate ID-12 was found to be the second most effective control agent after *P. fluorescens* ID-3 both in *in vitro* studies as well as under greenhouse conditions. Elewa *et al.* (2011) tested five isolates of *Bacillus subtilis* namely BS-12, BS-14, BS-17, BK-1, BM-1, for control of root diseases of sesame caused by *M. phaseolina*. *In vitro* studies revealed that in case of dual culture technique, the highest mycelial inhibition against *M. phaseolina* occurred with *B. subtilis* BS-17 (up to 65.56%). Enhanced plant growth in addition to disease control was achieved with *Pseudomonas* and *Bacillus* (Whipps, 2001; Sharma *et al.*, 2010). Siderophores have been shown to play a role in increased growth response of certain plants along with reduction of plant pathogen (Whipps, 2001). Mechanisms of biological control with bacterial strains are believed to include bacterial metabolites that adversely affect the pathogen or induce systemic resistance (Kloepper *et al.*, 1999). Various strains of species *B. amyloliquefaciens*, *B. subtilis*, *B. pasteurii*, *B. cereus*, *B. pumilus*, *B. mycoides*, and *B. sphaericus* are demonstrated to be potential ISR elicitors and significantly reduce the incidence or severity of various diseases on diverse hosts (Choudhary and Johri 2008; Kloepper *et al*., 2004). It is believed that plants have the ability to acquire enhanced level of resistance to pathogens after getting exposed to biotic stimuli provided by many PGPRs and this is known as rhizobacteria mediated ISR (Choudhary *et al*., 2007).

P. polymyxa is known to produce two types of peptide antibiotics, one type is only active against bacteria and the other is active against fungi, gram positive bacteria and actinomycetes (Beatty and Jensen, 2002). This specie also synthesizes plant hormones auxin (Lebuhn *et al*., 1997) and cytokinin (Timmusk *et al*., 1999). *P. polymyxa* strains are capable to produce several hydrolytic enzymes that play an important role in the biocontrol of plant pathogens (Helbig, 2001; Yang *et al*., 2004). Sakurai *et al.* (1989) found that *P. polymyxa* produced an amylase of 48 kDA that comprises 1,161 amino acids. In our study, *P. polymyxa* has suppressed the *X. campestris* pv. *sesami* which shows that it may be applied in combination with other antagonistic agents to get even better results.

The effective application of antagonistic bacteria to suppress the pathogenic microorganisms has been found an alternate to chemicals and also found an environmentally safe. The PGPRs have huge potential to assist the human being in fighting the disease pathogens until and unless extensive studies are carried out to explore their potential.

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