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Differentiation of the Pathotypes in Race 29 of Cotton Bacterial Leaf Blight Pathogen *Xanthomonas axonopodis pv. malvacearum (Xam)* and the reaction of some transgenic (*Bt*) and non-transgenic cotton varieties grown in the Vidharbha region to the prevalent *Xam* races and pathotypes

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ABSTRACT

Bacterial blight disease on cotton crop caused by the bacterial pathogen *Xanthomonas axonopodis pv. malvacearum* (*Xam*) is a serious disease found to infect both the transgenic (with *Bt* gene) and non-transgenic cotton varieties grown in the Western Vidharbha region of Maharashtra state, India. Different races of the *Xam* pathogen exist in the infected cotton crop. Their knowledge can be useful in breeding cotton varieties resistant to these races for the region. Similarly, the knowledge of the sensitivity of these *Xam* races to the various pesticides is useful in chemical disease management programs. At least 4 races of *Xam* were isolated from both transgenic (*Bt*) and non-transgenic cotton crops grown in this region. These races included race-7, race-29, race-30, and race-32 of the *Xam* pathogen. Further, the race-29 was differentiated into pathotype-A and pathotype-B based on their differential reaction on additional two differential cotton cvs viz. Gregg and DPX_4. This is the first report of the presence of pathotypes in *Xam* race-29. This is also the first report on how to differentiate the pathotypes in *Xam* race-29. No pathotypes in other *Xam* races were detected by using these two differentials. Transgenic and non-transgenic cotton crop varieties vary in their susceptibility (based on diseased water-soaking (WS) reaction, or resistant hypersensitive (HR) reaction) to these *Xam* races. Further, these races vary in their virulence (time required for the production of diseased reaction) on the same cotton genotypes. The infection phenomenon of *Bt* and non-*Bt* versions of cotton varieties was observed to be *Xam*-race specific. These *Xam* races did not differ in their sensitivity to a given pesticide. Antibiotics and a copper group of fungicides were effective in checking the growth of these *Xam* races under in vitro testing. The formation of pesticide-resistant mutants was not observed in these *Xam* races in this region. Cotton crop varieties (*Bt* transgenic and their non-*Bt* version) resistant to these races have to be bred for cultivation in this region to minimize the losses due to this disease.

Keywords: Cotton, Bacterial blight, Races, Pathotypes, Transgenic, *Bt* and non-*Bt* cotton, pesticides.

INTRODUCTION

Cotton crop (Gossypium spp) also known as white gold in the Indian sub-continent is grown in several countries of Asia region, parts of the USSR, some European countries, the American sub-continent, and in some African countries (Khan *et al.,* 2020). The fabrics and textile industry of the world is dependent on the production of this crop. The area under cotton crop in the world is around 32,500 million hectares with a production of 25 million tons (Amrouk and Palmeri**.**, 2021) which comprises both transgenic *Bt* cotton and non-*Bt* cotton crop varieties. In India, it is cultivated on an acreage of 11.9 million hectares (Minhas, 2023) with an annual production of 34.4 million bells (Anonymous, 2022a) having US$ 719.03 million in revenue for the nation (Anonymous, 2022b).

Bacterial blight caused by *Xanthomonas axonopodis pv. malvacearum (Xam)* is an important disease of cotton crops cultivated in most parts of the world (Jalloul *et al,* 2015) including India (Verma *et al,* 1974., Borkar

and Yumlembam, 2016). In India, the occurrence of bacterial blight disease in cotton fields in early 1970 has led to the development of resistant varieties to this disease (Verma, 1986). However, the prevalence of high variability in the bacteria pathogen *Xam* in the form of 32 races in India (Verma and Singh, 1975) made it an unfinished task for the breeder and pathologist to breed the region-cum-race specific varieties. Further, the development of new pathotypes in some of the *Xam* races is a matter of concern for the newly developed cotton crop varieties including the transgenic *Bt* cotton varieties. Both *Bt* cotton and non-Bt cotton are found susceptible to *Xam* (Sajid *et al,* 2018) which may depend on the prevalent *Xam* race. During the present investigation, we assessed the prevalence of different *Xam* races in the Vidharbha region of Maharashtra state, India; their disease-inducing capacity on transgenic (having *Bt* gene) and non-transgenic cotton crop varieties grown in this region; and the reaction of field prevalent *Xam* races to different pesticides molecules.

MATERIALS AND METHODS

Infection of bacterial blight pathogen *Xanthomonas axonopodis pv. malvacearum* (*Xam)* on transgenic *Bt* and its non-*Bt* version in cotton crop in Vidarbha region:

The cotton hybrids transgenic RCH-2(*Bt*), KDCH-20(*Bt*), and Bunny (*Bt*) with their non-transgenic version i. e. RCH-2, KDCH-20, and Bunny were found infected with the bacterial blight disease in the cotton growing belt of the Vidharbha region of Maharashtra state, India. The natural infection showed the angular water-soaked legions as well as the leaf veins blight due to infection **(Fig.1).** The infected leaf samples were brought to the laboratory for isolation of the causal bacterial pathogen

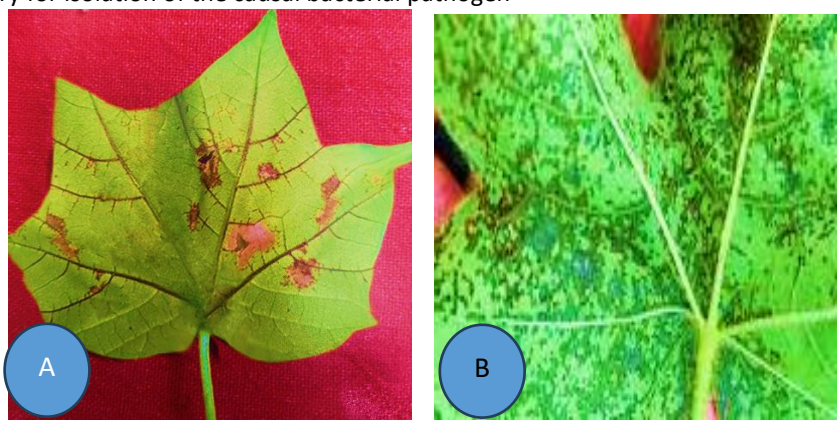

Fig.1. Natural infection of bacterial blight Pathogen *Xam* on cotton leaf showing (a) vein blight and (b) Leaf spot symptoms.

Isolation of Bacterial leaf blight pathogen of cotton:

The collected leaf samples of transgenic *Bt* and non-*Bt* versions of these cotton varieties were washed in running tap water to remove the dust particles and microbial epiphytes present on these leaves. These were pressed in sterilized blotter paper for drying and small pieces of diseased portions from the leaves were cut with the help of a sterilized scalpel, and bacterial isolation was made by the routine procedure of isolation of leaf spot invading bacterial pathogen (Borkar, 2017) on sterile Nutrient-Agar-Sucrose (NAS) medium in petri-plates.

The bacterial colonies having translucent, yellow, smooth, raised growth which developed after 72hrs of incubation **(Fig. 2)** were purified by streak plate method **(Fig.3).** A single purified colony with the above characteristics was transferred on NAS medium slants and incubated in BOD growth chamber at 29+1^{0}C temp for their growth. The cultures obtained from such individual plant variety isolation were designated with numbers. The young fresh growth of each isolate was tested for their pathogenicity on the universally susceptible cotton cv. Acala-44 to prove the pathogenicity of the isolated bacterial cultures.

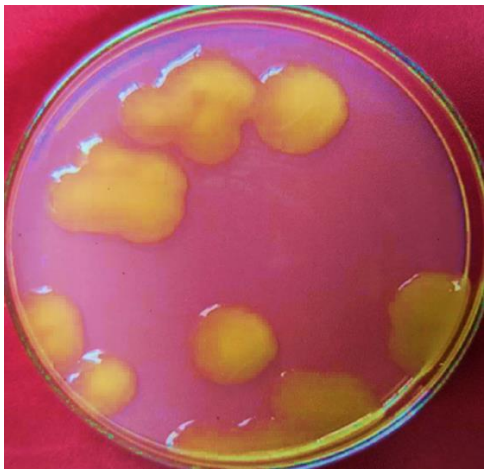

Fig. 2. Isolated *Xam* colonies from infected cotton leaf.

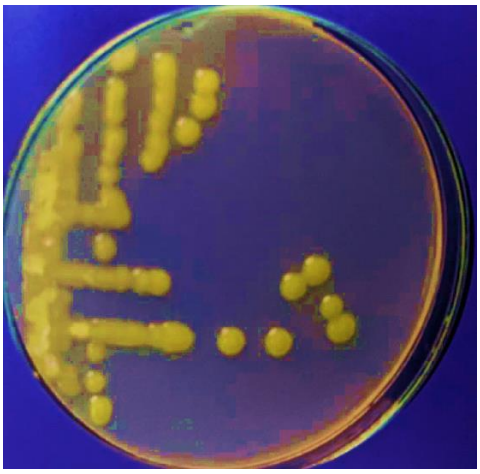

Fig. 3. Purification of *Xam* culture by using a single bacterial colony.

Pathogenicity Test of the isolated bacterial cultures:

To prove the pathogenicity of the isolated bacterial cultures from cotton plants, the pure cultures of the bacterium obtained from single colonies were used. The young growth of 48 hrs of bacterial cultures on nutrient-agar media was taken with a sterile scalpel and suspended in distilled sterile water; the OD of the bacterial suspension was adjusted to 0.1 OD at 620 nm in spectronic-20 spectrophotometer, so as to obtain the bacterial concentration of 10^7cfu/mL of water (Borkar,1990). The bacterial suspension was syringe infiltrated on the dorsal side of cotton leaves of susceptible cotton cv Acala-44. The inoculated plants were kept in a glass house at 28^0C temperature and the development of a susceptible water-soaking reaction **(Fig. 4)** induced by the bacterial culture was noted on the 3rd day of inoculation and further steps to prove Koch's postulate were followed.

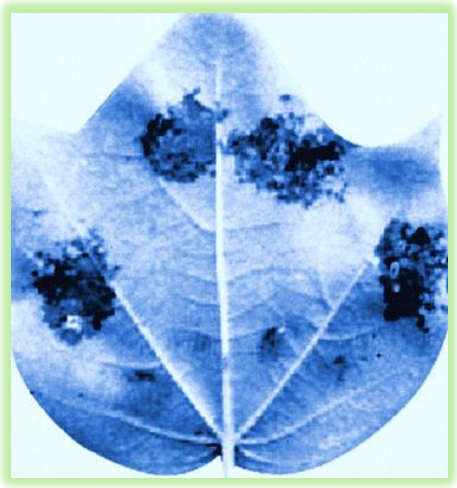

Fig.4. Disease (water-soaking) reaction developed by *Xam* cultures in syringe-infiltrated areas on cotton leaves.

Identification of races of bacterial blight pathogen *Xam* of cotton:

The protocol given by Verma and Singh (1975) was adopted for the identification of the races of *Xam*. For this race identification 7 cotton differentials viz. Acala-44, Stonevil-20, Stonevile-2BS9, Mebane- B-1, 1-10B, 20-3, and 101-102 B were obtained from the central cotton research institute, Nagpur and were grown in plastic pots in the glasshouse. At the 5-leaf stage, the leaves of these differentials were inoculated with individual bacterial suspension (0.1 OD at 620nm) by the syringe infiltration method (Borkar, 2017). After 3 days, the reaction in bacterial infiltrated areas was recorded (susceptible disease reaction= translucent water-soaking areas; Resistant reaction= hypersensitive browning reaction (HR) and Immune= no reaction in the bacterial infiltrated area) as HR is developed within 24 hrs while water-soaking disease reaction is developed after 3 days.

Differentiation of Pathotypes in *Xam* race 29:
Two additional cotton differentials viz. Gregg and DPX_4 were used to differentiate the pathotypes in race-29. The plants of these differentials were raised in the glasshouse in pots and 3 different isolates of race-29 (isolated from three different cotton varieties) were syringe inoculated, as above, in the leaves of these differentials, and reactions were recorded.

Virulence/Aggressiveness of *Xam* races on genetically modified *Bt* and non-*Bt* cotton hybrids:
Two cotton varieties viz.RCH-2 and Bunny with their *Bt* and non-*Bt* version were used for the determination of the aggressiveness of *Xam* races. The plants of these varieties were raised in the research farm of the Department of Plant Pathology, MPKV, Rahuri. Races of *Xanthomonas axonopodis pv. malvacearum(Xam)* isolated from *Bt* and non-*Bt* cotton varieties were used for aggressiveness studies. The suspension of these races was inoculated by syringe infiltration method into the leaves of the above varieties and plants were observed daily to record the disease symptoms appearance. Based on days required for induction of diseased water-soaking reaction on cotton hybrids viz. RCH *Bt*, RCH non-*Bt*, Bunny *Bt,* and Bunny non-*Bt* the virulence/aggressiveness of these races were determined.

Reaction of *Bt* and non-*Bt* cotton hybrids to different races of *Xam* :
To study the reaction of *Bt* and non-*Bt* cotton varieties to different races of *Xam*, 4 cotton varieties viz. RCH-2, Mallika, Bunny, and NHH-44 with their *Bt* and non-*Bt* version were grown in the research farm of the Department of Plant Pathology, MPKV, Rahuri. Different races of *Xam* isolated from *Bt* and non-*Bt* cotton varieties were inoculated by the syringe infiltration method into the leaves of the above varieties. After 3 days of inoculation, the disease water-soaking reaction/ hypersensitive resistance reaction of cotton leaves to bacterial races was recorded.

Sensitivity of different *Xam* races to various pesticides:
Poison food technique was used to study the sensitivity of different races of *Xam* to different fungicides viz. Thiram, captan, Mancozeb, Carbadazim, Sulfur, Chlorothalonil, Propiconazole, Fusilazole, Copper-oxychloride, and copper hydroxide; bactericides viz. Streptocycline, Streptomycin, Bacteriocin, and insecticides Thimethoxam and Endosulphan. Nutrient agar media containing fungicides (at 0.2 % conc.), bactericides (at 100 ppm conc.), and insecticides (at 0.2% conc.) were used. The culture of *Xam* races was plated on these mediums to test their sensitivity against these fungicides, bactericides, and insecticides. These plates were incubated in BOD at 29 ^{0}C for 3 days and observations were recorded for the effect of pesticides on the *Xam*. Those cultures that grew on the pesticide-containing plates were tested for their pathogenicity on the susceptible cotton cv. acala-44.

RESULTS

1. **Prevalence of *Xam* races in western Vidharbha cotton tract and the reaction of cotton varieties grown in the region to these races:**

Three cotton varieties having the transgenic background of the *Bt* (*Bacillus thuringenesis* gene) gene with their non-*Bt* version were found infected in the cotton crop fields of the Vidharbha region. These varieties included RCH-2 (*Bt* and non-*Bt*), KDCH-20 (*Bt* and non-*Bt*), and Bunny (*Bt* and non-*Bt*). These varieties were infected by 4 different *Xam* races in this region (table 1). RCH-2 of *Bt* and non-*Bt* version was found infected with *Xam* race-29. KDCH-20, non-*Bt* was also infected with *Xam* race-29, while its *Bt* version was infected with *Xam* race-7. The Bunny non-*Bt* crop was infected with *Xam* race-32 while its *Bt* version was infected with *Xam* race-30.

Table 1. Identification of prevailed *Xam* races isolated from infected transgenic- *Bt* and non-Bt cotton varieties from western Vidharbha region.

Isolate No	Isolated from Infection on	Reaction on cotton differential used for *Xam* race identification							Race No
		Acala-44	Stoneville 2B S9	Stoneville 20	Mebane B-1	1-10B	20-3	101-102B	
1	RCH-2 *Bt*	**+**	**+**	**+**	**-**	**+**	**+**	HR	**29**
2	RCH-2 non-*Bt*	**+**	**+**	**+**	**-**	**+**	**+**	HR	**29**
3	KDCH-20 *Bt*	**+**	**+**	**+**	**-**	HR	**-**	HR	**7**
4	KDCH-20 non-*Bt*	**+**	**+**	**+**	**-**	**+**	**+**	HR	**29**
5	Bunny *Bt*	**+**	**+**	**-**	**+**	**+**	**+**	HR	**30**
6	Bunny non-*Bt*	**+**	**+**	**+**	**+**	**+**	**+**	HR	**32**

+ = susceptible water-soaking diseased reaction, HR**=** Hypersensitive browning reaction;
- = Immune (no reaction).

The prevalence of different *Xam* races in this region and their varietal-specific disease-inducing ability suggests for growing of cotton varieties resistant to these 4 races in this region.

2. Detection of pathotype in *Xam* race-29:

All the *Xam* races prevalent in this cotton growing tract were inoculated on two additional cotton differentials to assess the presence of variability/pathotype within the race. The results (table 2) indicated that *Xam* race-29 differed in their disease-inducing reaction on these two additional cotton cvs. Race 29-A was able to infect the cotton cv. Gregg and DPX_4; whereas race 29-B was unable to infect the cotton cv. DPX_4. Therefore, to differentiate *Xam* race-29, the additional cotton cvs. Gregg and DPX_4 should be used.

All other prevalent races viz. race-7, race-30, and race-32 have the same reaction on these two additional cvs, and therefore there was no pathotype present in these races except race-29.

Table 2. Differentiation of *Xam* race-29 in pathotypes based on their reaction to two additional cotton differentials.

Xam Isolate no	Isolation source	*Xam* Race no.	Reaction on additional Cotton cv Gregg	Reaction on additional cotton cv DPX_4	Pathotype of *Xam* race
1	**RCH-2 transgenic Bt**	**29**	**+**	**+**	**29-A**
2	**RCH-2 non-transgenic**	**29**	**+**	**+**	**29-A**
4	**KDCH-20 non-transgenic**	**29**	**+**	**-**	**29-B**

3. Does *Bt* gene increase the susceptibility of cotton variety to *Xam* races:

It is apparent from the result (table 3) that the incorporation of *Bt* gene in the cotton hybrid varieties viz. RCH-2 and Bunny increased the susceptibility (time to induce the disease reaction) of the variety to certain *Xam* races. *Xam* race-29-A, race-30, and race-32 were more virulent on *Bt* cotton than its non-*Bt* version for RCH-2 and Bunny. The disease reaction induction time was decreased by 24 hrs in the *Bt* cotton. This decrease in disease induction time, in the favorable atmospheric environment, may increase the inoculum load for the disease spread and infection.

Table 3. Virulence of *Xam* race on some of the cultivated transgenic-*Bt* and non-transgenic cotton varieties.

Race no/ Pathotype	Time required for induction of disease water-soaking reaction (in days) on cotton varieties			
	RCH-2		Bunny	
	***Bt* cotton**	**non-*Bt* cotton**	***Bt* cotton**	**non-*Bt* cotton**
7	6	HR	-	HR
29-A	5	**6**	5	**6**
29-B	6	HR	HR	HR
30	4	**5**	4	**5**
32	4	**5**	4	**5**

4. **Comparative reaction of commercial *Bt* and non-*Bt* cotton varieties to prevalent *Xam* races:**

Four cotton hybrids with their *Bt* and non-*Bt* version were tested against 4 *Xam* races including pathotypes of *Xam* race-29. The reaction of these varieties was dependent on the *Xam* race-cotton hybrid (*Bt* and non-*Bt* version) interaction (table 4). Cotton hybrid Mallika with its *Bt* and non-*Bt* versions was resistant to the prevalent 4 races in this geographical region. RCH-2 non-*Bt* version was resistant to race-7 and race-29-B only. Its *Bt* version on the other hand was susceptible to all the prevalent races. The *Bt* and non-*Bt* versions of NHH-44 and Bunny were susceptible to race-29-A. The *Bt* version of NHH-44 was susceptible to race-30 and 32 while its non-*Bt* counterpart was resistant. These results indicate that the integration of the *Bt* gene in the cotton hybrid changed the cotton variety from bacterial blight resistant to bacterial blight susceptible for some races.

Table 4. The comparative reaction of transgenic-*Bt* and non-transgenic cotton varieties to prevalent *Xam* races and their pathotypes in Western Vidarbha.

Race no	Reaction on commercial *Bt* and non-*Bt* cotton varieties							
	Mallika		RCH-2		NHH-44		Bunny	
	Bt	**non-*Bt***	***Bt***	**non-*Bt***	***Bt***	**non-*Bt***	***Bt***	**non-*Bt***
7	HR	HR	+	HR	HR	HR	-	HR
29-A	HR	HR	+	+	+	+	+	+
29-B	HR	HR	+	HR	HR	HR	HR	HR
30	HR	HR	+	+	+	HR	+	+
32	HR	HR	+	+	+	HR	+	+

HR=hypersensitive resistant browning reaction, + = diseased reaction, and - = no reaction (immune reaction)

5. Reaction of prevalent *Xam* races and pathotypes to pesticide molecules:

The reaction of 4 *Xam* races including 2 pathotypes were assessed under *in vitro* conditions against different fungicides, insecticides, and antibiotics used in the cotton crop production system. Under the *in vitro* test (table 5) the growth of all the races was either inhibited or not inhibited by a particular pesticide. There was no difference among these races towards their reaction to a given pesticide. Among the pesticides, Thirum, Captan, Mancozeb, Copper-hydroxide, Copper-oxy-chloride, and antibiotics inhibited bacterial growth and can be useful in the disease management program. Other pesticides did not inhibit bacterial growth nor induce the pesticide-resistant mutant in these races.

Table 5. In vitro reaction of *Xam* races and pathotypes to pesticide molecules.

Name of pesticide molecule	Sensitivity of *Xam* races				
Fungicides (0.2%conc.)	**7**	**29A**	**29B**	**30**	**32**
1.Thirum	-	-	-	-	-
2.Captan	-	-	-	-	-
3. Carbendazim	+	+	+	+	+
4.Sulfur	+	+	+	+	+
5.Mancozeb	-	-	-	-	-
6.Cholothalonil	+	+	+	+	+
7.Propiconazole	+	+	+	+	+
8.Fusilazole	+	+	+	+	+
9.Difenconazole	+	+	+	+	+
10.Copper-hydroxide	-	-	-	-	-
11.Copper-oxychloride	-	-	-	-	-
12.Aureofungin	-	-	-	-	-
Insecticides (0.2%conc)					
1.Spark	+	+	+	+	+
2.Endosulfon	+	+	+	+	+
3.Thimethoxam	+	+	+	+	+
Antibiotics (100 ppm conc.)					
1.Streptocycline	-	-	-	-	-
2.Sterptomycin	-	-	-	-	-
3.Bacteriocin	-	-	-	-	-

+ = non-sensitive (growth of *Xam* on chemical plates); - = sensitive (no growth of *Xam* on chemical plates)

DISCUSSION

The prevalence of various pathogenic races of a particular crop bacterium in a region demands a horizontal resistance variety of a crop (Borkar, 1990; Bossa-Castro *et al,* 2018) to restrict the invasion of concerned races of the bacterium. In the cotton-growing tract of Vidharbha region of Maharashtra state, at least 4 races of cotton bacterial blight pathogen *Xam* were present to cause bacterial disease in cotton varieties that were susceptible to these individual/ group of *Xam* races. RCH-2 (*Bt* and non-*Bt* crop), and KDCH-20 (non-*Bt crop)* were infected with *Xam* race-29, while KDCH-20 *Bt* crop was infected with *Xam* race-7. The Bunny non-*Bt* crop was infected with *Xam* race-32 while its *Bt* version was infected with *Xam* race-30. These results are suggestive of considering the prevalence of *Xam* races in a particular cotton growing tract before a selection of a cotton variety for cultivation and the availability of resistance to the prevalent races of *Xam* in that variety. Verma and Singh (1975) reported the prevalence of different *Xam* races among the cotton growing tracts in various states/provinces of India and is a concern for cotton growers to have a resistance variety for the available pathogenic virulence in that cotton growing tract. In India, a total of 32 races of *Xam* are present (Verma, 1970) and their presence varies in different states (Kumar, 2018). However, 19 *Xam* races have been described in different parts of the world (Hunter, 1968) and *Xam* race 18 is the most virulent and occurs predominantly throughout the world (Thaxton *et al,* 2001). Other races include races 20, 21, and 22 reported from Africa (Follin *et al,* 1988). In India, Meshram *et al* (2002) reported 14 different races such as races 1-8, 10, 11, 12, 13, 15, and 18 from major cotton growing regions, while Gholve *et al* (2005) reported race 18 and 7 from Marathwada region of Maharashtra. From our results, it is evident that *Xam* race 7, 29, 30, and 32 were present in the Vidharbha region of Maharashtra state.

Various workers reported the presence of races in *Xam* bacterium in different parts of the world. Hussain (1984) reported variation in *Xam* races for Pakistan; Bird and Tsai (1975) and Thaxton *et al* (1992) for the USA; Verma and Singh (1975) for India; Follin *et al* (1988) for Africa etc, indicating that the bacterial pathogen *Xam* have different virulence and races. Identification of races in bacterial plant pathogens is an important aspect of knowing the variability in the particular bacterium (Borkar, 2017). The presence of races

and the scheme for their identification was reported for bacterial plant pathogens particularly *X. c. pv. malvacearum* (Hunter et.al, 1968., Verma and Singh, 1975); *X. c. pv. campestris* (Vincente *et.al,* 2001; Fargier and Manceau,2007); *X. oryzae pv .oryzae* (Hoang et.al, 2007), *X. axonopodis. pv. viticola* (Borkar, 2002), *Pseudomonas solanacearum* (Buddenhagen *et al,* 1962; Pegg and Moffett, 1971; He *et al,*1983), and *Ralstonia solanacearum* (EPPO,2004). The presence of pathovar in the races is only reported for *X.c.pv. campestris* (Vincente et.al, 2001; Fargier and Manceau, 2007). Now in this paper, we report the presence of pathovar in *X. c. pv. malvacearum* race-29 and the scheme for their differentiation.

Transgenic *Bt* cotton is grown over a large area in many countries (James, 2016; Rocha-Munive *et al.,* 2018). However, their extent of susceptibility to different *Xam* races has not been studied so far. Our studies indicated that the incorporation of *Bt* gene in the cotton hybrid varieties viz. RCH-2 and Bunny increased the susceptibility (time to induce the disease reaction) of the variety to certain *Xam* races. *Xam* race-29-A, race-30, and race-32 were more virulent on *Bt* cotton than its non-*Bt* version of RCH-2 and Bunny. The disease reaction induction time was decreased by 24 hrs in the *Bt* cotton. This decrease in disease induction time, in the favorable atmospheric environment, may increase the bacterial inoculum load for the disease spread and infection. Further, the *Bt* and non-*Bt* cotton crop varieties reacted differently to different *Xam* races. No such study is available at present. In the management of bacterial blight infection, pesticide and antibiotic molecules play an important role (Taylor and Reeder, 2020). However, sensitivity variation for different pesticide molecules within the races of a pathogen is an important issue. Variation in the sensitivity/reaction of races to the same pesticide was reported for *X. a. pv. puniceae* (Raghuwanshi *et al,* 2013) and *X. c. pv. viticola* (Borkar *et al,* 2017) in Maharashtra state. The *Xam* races and pathotypes under study were sensitive to some pesticides and not to others. There was no variation among the races in the sensitivity to a single pesticide.

CONCLUSION

These results have significance for the development of cotton varieties (both *Bt* and non-*Bt*) resistant to bacterial blight pathogen for a particular cotton growing tract/region based on the presence of *Xam* races in that region. Further, the knowledge of the sensitivity of the *Xam* races present in the region to different pesticide molecules is useful and can be utilized in the management of bacterial blight pathogen of the cotton crop.

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