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Research Article

Synergizing Genetic Resistance and Antibiotics to Combat *Xanthomonas* Blight in Upland Cotton

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ABSTRACT

Cotton (*Gossypium hirsutum*) is an important crop in Pakistan that is used to get fiber and many other industrial products that are helpful in making the country's economy better. Cotton is attacked by the *Xanthomonas axonopodis* pv. *malvacearum (Xam)* causes the bacterial blight disease, which is a major worldwide cause in every country. Five different isolates of *Xam* were used after biochemical characterization and 48 cotton genotypes were screened in the field against *Xam* and evaluated on the basis of morphological and physiological traits. Out of 48 cotton genotypes, none showed an immune response, only 3 genotypes showed a resistant response, 13 genotypes showed a tolerant response, 24 were susceptible to *Xam* and 7 genotypes were highly susceptible. Two different antibiotics, Tetracycline and Ampicillin were also tested *in vitro* on 5 isolates as well as *In planta* against one susceptible genotype at six different concentrations for their efficacy. *In vitro* test was checked through a spectrophotometer. Tetracycline was found to be most effective even at low concentrations. The susceptible genotype (CIM-591) showed a tolerant response on the application of tetracycline at low concentrations in the field but showed resistance at high concentrations.

Keywords: Cotton (*Gossypium hirsutum*), Bacterial Blight Disease, *Xanthomonas axonopodis* pv. *malvacearum* (*Xam*), Antibiotics, Tetracycline, Resistance

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INTRODUCTION

Cotton (*Gossypium hirsutum*) belongs to the family Malvaceae and genus *Gossypium* (Wendel and Grover, 2015). Cotton is broadly adapted to growing in temperate, subtropical and tropical environments, but its growth can be influenced by climate conditions, including temperature and water availability (Jans *et al.,* 2021). Around 80% of cotton is used in the clothing industry, making it a staple for garments like t-shirts and jeans. About 15% of cotton is used in home furnishings, including bed linens, curtains and upholstery. The remaining 5% is used in products like medical supplies, filters and padding (News, 2021). Cottonseed oil is rich in unsaturated fatty acids, particularly linoleic acid, which is beneficial for heart health. It also contains tocopherols, which have antioxidant properties. Cottonseed meal, a byproduct of oil extraction, is high in protein and can be used as animal feed. It contains essential amino acids, making it a valuable protein source. One of the challenges with cottonseed is the presence of gossypol, a toxic compound that can limit its use in food and feed. However, research is ongoing to develop gossypol-free or low-gossypol cotton varieties (Ashokkumar and Ravikesavan, 2011).

For the 2022/23 season, global cotton production was estimated at 117 million bales. The total area harvested worldwide was approximately 32.3 million hectares. The leading producers were India: 27.5 million bales, China: 26.5 million bales, United States: 15.5 million bales and Brazil: 13.8 million bales (Meyer and Dew, 2022). Pakistan ranked 5th in the production of cotton worldwide. In Pakistan, the cotton production for the 2022/23 season was significantly impacted by severe flooding. The production was estimated at 5 million bales. The area harvested was reduced to 1.79 million hectares due to the flooding (Sheikh, 2022). Bacterial blight of cotton also known as angular leaf spot, seedling blight, or black arm which is caused by *Xanthomonas axonopodis* pv. *malvacearum* (formerly referred to as *Xanthomonas axonopodis* pv. *malvacearum* and *Xanthomonas malvacearum*) (Showmaker *et al.,* 2017). Bacterial blight caused by *Xanthomonas axonopodis* pv. *malvacearum* can lead to significant yield losses in cotton up to 1% to 27% under typical conditions. In some extreme cases, yield losses have been reported to reach as high as 80% (Manna *et al.,* 2024). In severe outbreaks, especially under favorable conditions for the bacterium (warm and humid weather), losses can exceed 50% (Mijatović *et al.,* 2021).

Bacterial blight of cotton, caused by *Xanthomonas axonopodis* pv. *malvacearum*, presents as small, angular, water-soaked lesions on leaves, often surrounded by yellow halos. These lesions can enlarge, turn black and cause premature defoliation. On stems and petioles, black cankers, known as “black arm,” can girdle and kill parts of the plant. Bolls exhibit water-soaked spots that become sunken and dark brown or black, leading to internal boll rot, lint discoloration and seed contamination. These symptoms collectively impact the plant’s health and yield (Kemerait *et al.,* 2017). Bacterial blight in cotton can originate from infected crop residue or seed. The bacterium survives in field debris and soil, with survival duration influenced by environmental conditions. Infections spread via wind-driven rain, irrigation and contaminated equipment. Bacterial blight in cotton, caused by *Xanthomonas axonopodis* pv. *malvacearum*, is more severe when it develops early in the season, particularly if seedlings are infected. The bacterium can enter through stomates, lenticels, hydathodes and wounds caused by wind-blown sand. Once established, rainfall shortly after planting can lead to rapid spread. After canopy development, heavy rainfall followed by warm, humid conditions (relative humidity >85%) further increases disease spread. Favorable conditions for bacterial blight include daytime temperatures of 90-100°F and nighttime temperatures of 62-68°F. Under these conditions, even one infected seed out of 6,000 can start an epidemic (Kemerait *et al.,* 2017).

The first report of bacterial blight of cotton caused by *Xanthomonas axonopodis* pv *malvacearum* in Pakistan was documented in 1986 (Manna *et al.,* 2024). This disease, which significantly impacts cotton yield, was initially identified in Alabama, USA, in 1891 (Jalloul *et al.,* 2015). Bacterial blight, caused by *Xanthomonas citri* subsp. *malvacearum* (*Xcm*), is a constant threat to cotton production globally, causing severe yield loss and economic injury (Verma, 1986; Brinkerhoff, 1970). Traditional management methods, such as the use of resistant varieties, seed treatment, and cultural practices, have achieved varying degrees of success (Wang *et al.,* 2018). New races of the pathogen and breakdown of resistance genes further hinder long-term control (Delannoy *et al.,* 2005). Antibiotics have come under consideration in this respect because they can inhibit the growth of bacteria directly, and they could be a useful weapon for the suppression of disease (McManus *et al.,* 2002). The use of general antibiotics for the control of *Xcm* in cotton has not been tested extensively, particularly under field conditions. Knowledge about their effectiveness is vital in formulating integrated disease management with the integration of chemical, cultural, and genetic practices.

In addition, studying the interaction between host plant response and antibiotics could lead to the creation of resistant cultivars and provide insight into pathogen suppression at the molecular level (Ryan *et al.,* 2011). The objective of this study was to screen commonly grown different genotypes of cotton based on physiological and morphological parameters of normal and diseased plants. To check the efficacy of two antibiotics on different isolates of *Xam* and also the field evaluation of these antibiotics on susceptible variety. Tetracycline and Ampicillin were selected because of their established effectiveness against Gram-negative bacteria such as *Xanthomonas* spp. in previous research (Khan *et al.,* 2012; Sarker *et al.,* 2019). Their cost-effectiveness and broad-spectrum activity render them potential candidates for field use, although risks of resistance require further investigation (Abdelraheem *et al.,* 2024).

MATERIALS AND METHODS

Isolation from infected samples

Cotton leaves with clear bacterial lesions were collected from various fields and brought to the laboratory. Leaf spots were cut into small pieces, disinfected in 70% ethanol for 30 seconds, rinsed in distilled water and placed on sterile tissue paper to remove excess moisture (Clifton, 1958). Nutrient Agar (NA) was used for the isolation, purification and multiplication of the *Xanthomonas axonopodis* pv *malvacearum* bacteria. Media was prepared by using NGA recipe (Beef Extracts 3g, Glucose 3g, Peptone 5g, NaCl 5g, Agar 15g and Distilled Water up to 1 Liter), autoclaved and

poured into the sterilized petri plates on lukewarm condition in the clean laminar flow chamber and leave it to solidify. The dried samples were then transferred to Petri plates in a laminar flow chamber and incubated at 30°C for 24 hours. Bacterial growth was observed, and after biochemical testing of forty samples, five *Xam* isolates were selected for further experiments.

Purification and identification of bacterium

The isolated bacterium formed dark yellow, round colonies on nutrient agar and was motile with a single flagellum. It fermented sucrose, glucose, maltose, and lactose, producing acid (Dowson, 1957) and exhibited gram-negative reactions, indicating it belonged to the genus *Xanthomonas*. When pure colonies were fully grown on the Petri plate, an aqueous suspension of bacterium 10^8 cfu/ml was prepared by the dilution plate technique method in the nutrient broth media.

Biochemical tests for characterization

Gram staining

On the glass slide, a drop of distilled water was added. From a pure culture of bacteria, a sterile loop full of bacteria was removed, placed on the slide and fixed by heating. The slide was moved to the washing area. The bacterial culture that was put on the slide was treated with crystal violet for 60 seconds. After applying crystal violet, the slide was washed with distilled water, then treated with iodine. In the end, it was cleaned with ethanol for fifteen seconds. Safranin was used after washing with ethanol. The slide was placed under the microscope and visualized at 100X (Moyes *et al.,* 2009).

KOH test

Potassium Hydroxide (KOH) test was performed to confirm the Gram staining results. The bacterium from the pure culture plate was transferred to a glass slide having a drop of 3% KOH, stirred for 10 seconds in a circular motion by hand until the formation of slime thread-like appearance was observed (Ryu, 1940).

Screening of genotypes

A total of 48 genotypes were used to screen the *Xam* in the field. These genotypes were chosen on the basis of their applicability to current breeding programs, accessibility, and their documented or unsubstantiated response to bacterial blight in earlier field trials and literature (Abdelraheem *et al.,* 2024; Zhang *et al.,* 2020). The inclusion criteria focused on a wide coverage of genetic diversity, both known susceptible and resistant lines, to allow for exhaustive assessment of genotype-pathogen interactions.

These genotypes of cotton were sown in the field by using a Randomized Complete Block Design (RCBD) to evaluate it. Bacterial Blight of Cotton caused by *Xam* was observed on cotton plants under optimal weather conditions. The inoculum was already present in the field in sufficient amounts because this field was used previously against this pathogen. The rubbing method, artificial inoculation, is used to inoculate plants in the field when plants were 60 days old and 2-3 feet tall. All genotypes were screened by using the Brinkerhoff scale (Brinkerhoff, 1977) as shown in Table 1, classifying them as immune, resistant, tolerant, susceptible, or highly susceptible based on lesion production on plant leaves. Disease incidence was determined by the lesion counts and according to this all genotypes were graded. Symptoms of the disease appeared after 2 weeks of inoculation and it was observed that the disease became more severe after rain. This proves that rain and air flow spread this disease more.

Table 1. The scales were adopted according to the methodology of Brinkerhoff, (1977).

Grade	Symptom	Level
0	No macroscopic symptoms	Immune (I)
1-3	Round dry pinhead size lesions developed	Resistant (R)
4-6	Lesions turned to dry angular lesions	Tolerant (T)
7-9	Lesions turned to water-soaked spots	Susceptible (S)
10	Spots turned to large angular water-soaked lesions on leaf veins	Highly Susceptible (HS)

Note: The scale is based on visual symptoms and classifying genotypes with similar resistance response against the pathogen Xam in a pre-determined resistance level and give a quick view about the genotypes under screening.

In vitro* evaluation of different Antibiotics against *Xam

Two different antibiotics Ampicillin and Tetracycline were used against the OD growth of *Xam* isolates using suspension cultures in liquid growth media. The choice of tetracycline and ampicillin was determined by their established effectiveness against *Xanthomonas* spp., practicality in the field, and affordability. Tetracycline suppresses protein synthesis through 30S ribosomal binding, showing >90% inhibition against *Xam* at 100 µg/mL (Sarker *et al.,* 2019),

whereas ampicillin inhibits cell wall formation with 50-80% effectiveness (Talib *et al.,* 2020). These were used over streptomycin because of resistance issues in *Xam* (Abdelraheem *et al.,* 2024) and farmer affordability for their application in the field (Gholve *et al.,* 2024), and concentrations were maintained below 10 mg/mL as per FAO guidelines (Meyer and Dew, 2022).

Different concentrations of these antibiotics were applied against it. These concentrations were prepared from the stock solution of these antibiotics for the *in vitro* evaluation against *Xam*. These antibiotics were collected from the molecular biology lab and tested along with autoclaved double-distilled water as a control. Six different concentrations of both antibiotics were used with autoclaved double-distilled water as a positive control. These concentrations were prepared by diluting the stock solution in the ratio of 9:1 (9 parts from water and 1 part from the stock solution) up to six concentrations in the Eppendorf tubes, just like the serial dilution technique, resulting in six concentrations of each antibiotic, with a volume of 1 ml each.

In the lab, bacterial isolation and biochemical testing of possible *Xam* isolates were kept as glycerol stocks. The dilutions for the antimicrobial tests were made as previously stated. Under sterile conditions, 2 ml of the nutrient broth was inoculated with the bacterial stocks in test tubes. Once the test tubes were inoculated, they were shaken at 200 rpm for the whole night at 30°C. Using 200 µl of the bacterial culture as inoculum from the test tubes, fresh cultures of 20 ml each for each bacterial isolate were made the next day. They were then incubated at 30°C with 200 rpm shaking using nutritional broth of ½ strength. A spectrophotometer was used to measure the bacteria's optical density (OD) after three to four hours in order to make sure that young cells were being used for antibiotic testing. Testing was conducted on the bacterial cultures when the optical density (OD) at 600 nm reached 0.05.

Ninety-six well plates were used for the testing. 50 µl of the appropriate antibiotic dilution and 150 µl of bacterial solution with an OD of 0.05 at 600 nm were combined in each well. This procedure was carried out again for every possible combination of antibiotic dilutions. 200 µl of nutritional broth was used as a bacterial growth control and 50 µl of antibiotic-free broth was used as a drug control. Without any treatment, two wells were maintained as an additional control with bacterial growth. Every task that needed to be done in a sterile environment was done in a laminar flow cabinet. At 30°C, the plate was shaken at 200 rpm during incubation. After the plate was filled, readings were taken right away (0 hour) as a baseline and the impact of the antibiotics was noted 24 and 48 hours later. The Minimal Inhibitory Concentration (MIC 50), at which 50% of bacteria die, was calculated using these findings. For the second and third replication, the same process was used and the data were recorded (Sarker *et al.,* 2017).

***In planta* efficacy of antibiotics**

Both antibiotics (Ampicillin and Tetracycline) were applied to the susceptible genotype of cotton (CIM-591) in the field at six different concentrations. The application of these antibiotics was done by spraying them on the plants. The disease response was checked on the genotype as the symptoms appeared on the plants of cotton. Data from all treatments were recorded and analyzed.

Statistical analysis of the data

The data were analyzed using analysis of variance (ANOVA) under a completely randomized design with mean comparison following the least significant difference test (Steel *et al.,* 1997).

RESULTS AND DISCUSSION

Biochemical test for characterization

Gram staining

The Gram Staining procedure discovered that the bacteria were Gram-negative, as demonstrated by the red coloration of their cell wall on observation under a microscope. Gram-negative bacteria appear red or pink in color after Gram staining because of their cell wall structure. The cell walls of gram-negative bacteria are rich in lipids and a thin coating of peptidoglycan. Due to this peptidoglycan layer, gram-negative bacteria cannot hold the initial crystal violet stain, which was washed out by ethanol throughout this process. Following this, safranin counterstain was added, which was absorbed by the cell wall and gave it a reddish-pink color. Similar results have been reported by previous studies (Moyes *et al.,* 2009).

KOH test

Through a 3% KOH test, a distinct mucoid filament formation while using the inoculation loop on the slide indicates that the bacterial pathogen associated with bacterial blight of cotton was gram-negative bacteria. Similar results have been reported by previous studies (Waris *et al.,* 2016).

Screening of genotypes

Angular leaf spot of cotton had been an important cotton issue (Smith, 1920) and this is a reemerging disease in the recent times as well. To find out the sources of resistance, 48 genotypes of upland cotton were tested against *Xam* under natural environmental conditions, any of which were not free of *Xam* . Lesions on leaves and stems were the main disease symptoms that emerged during *Xam's* peak season. The standard disease rating scale was used to record each genotype's reaction to the bacteria (Brinkerhoff, 1977). Lesion production on the leaves of selected plants was recorded with their size of lesions to assess the resistance of genotypes against the disease as shown in Figure 1. From these results, it was shown that no genotypes of cotton were immune, 3 genotypes of cotton were moderately resistant, 13 genotypes were tolerant, 25 genotypes were susceptible, and 9 genotypes were highly susceptible to bacterial blight of cotton as shown in Tables 2 and 3. These results showed that genotypes of *G. hirsutum* is susceptible to *Xam*, but only a few genotypes show tolerance. Because there may be several harmful races of the bacterium in the field, this susceptibility is very problematic.

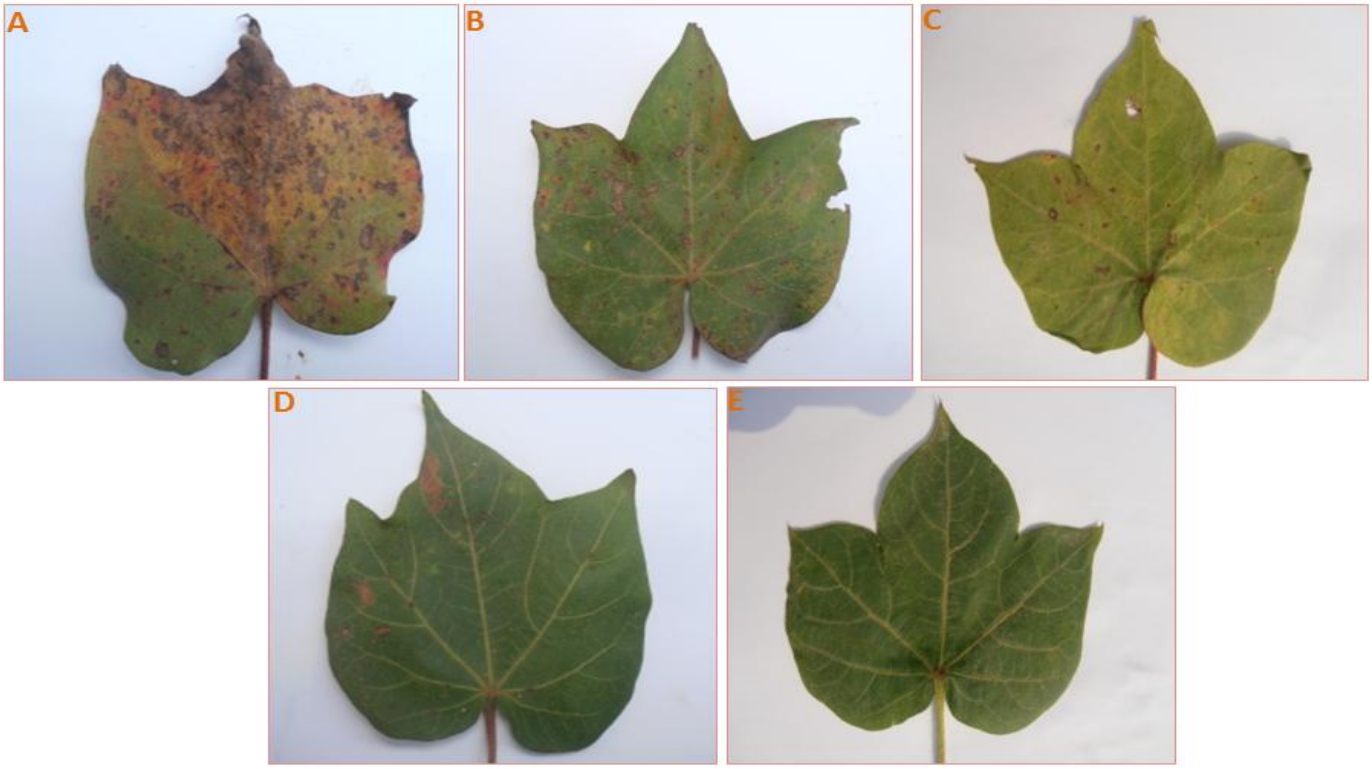

Figure 1: The leaves with varying symptoms of disease are shown. (A) shows a severe attack and it is marked as a highly susceptible genotype. Such lesions are also observed on the other vegetative parts of the plant and on the bolls as well. The leaves with lesions in (B) were marked as susceptible while in (C) the leaf symptoms present a tolerant plant type and (D) is resistant against *Xam*. Practically no variety was found immune. To make a comparison a leaf with no symptoms was assumed as the immune type shown in (E).

Table 2. Varietal response to bacterial blight of cotton under field conditions.

Sr. No.	Genotypes names/coded names	Response	Disease Rating
1	CIM-591	S	7
2	MS-95	S	7
3	CA-12	S	8
4	K	T	5
5	BS-52	S	9
6	A.A-905	R	1
7	MNH-506	S	8
8	BS-142	T	4

9	FH-1000	T	6
10	CM-602	HS	10
11	N	S	7
12	MS-289	R	3
13	FH-87	S	9
14	BS-988	S	8
15	MS-71	T	4
16	A.A-919	T	4
17	HUMA-15	S	7
18	L	S	7
19	NIAB-KREN	T	6
20	N-112	T	6
21	A.A-703	T	5
22	CM-599	S	9
23	Z-31	HS	10
24	P	T	5
25	FREGO BREET	S	7
26	VH-55	S	9
27	MNH-129	S	9
28	MS-39	T	5
29	O	HS	10
30	A.A-904	S	7
31	BS-815	S	9
32	FH-115	S	8
33	VH-53	HS	10
34	CM-595	S	9
35	I	HS	10
36	FH-94	S	7
37	MNH-700	S	7
38	VH-300	S	7
39	FH-900	S	8
40	H-499-3	HS	10
41	J	S	8
42	CM-496	T	5
43	M	T	5
44	MNH-93	T	4
45	FH-682	S	8
46	CM-608	HS	10
47	MNH-552	S	8
48	L-H-72	R	3

Table 3. Total number of lines according to Disease Gradings for Bacterial blight of Cotton.

Grade	Symptoms	Level	Lines	No of lines
0	No macroscopic symptoms	Immune (I)	-	-
1-3	Round dry pinhead size lesions developed	Resistant (R)	A.A-905, MS-289 & L-H-72.	3
4-6	Lesions turned to dry angular lesions	Tolerant (T)	V-K, BS-142, FH-1000, MS-71, A.A-919, NIAB-KREN, N-112, A.A-703, V-P, MS-39, CM-496, V-M & MNH-93.	13
7-9	Lesions turned to water-soaked spots	Susceptible (S)	CIM-591, MS-95, CA-12, BS-52, MNH-506, V-N, FH-87, BS-988, HUMA-15, V-L, CM-599, FREGO BREET, VH-55, MNH-129, A.A-904, BS-815, FH-115, CM-595, FH-94, MNH-700, VH-300, FH-900, V-J, FH-682 & MNH-552	25
10	Spots turned to large angular water-soaked lesions on leaf veins	Highly Susceptible (HS)	CM-602, Z-31, V-O, VH-53, V-I, H-499-3 & CM-608.	7
			Total	48

Some studies were conducted on the screening of cotton germplasm against bacterial blight of cotton. According to the screening trial conducted by Delannoy *et al.* (2005), cotton genotypes exhibit a hypersensitive reaction (HR) to *Xam*, which is triggered by resistance genes (R genes) and resistance gene analogs (RGAs). Additionally, it demonstrated that phytoalexins only build up in HR cells at antimicrobial concentrations, highlighting their critical function in pathogen containment (Delannoy *et al.,* 2005).

Previously, the glasshouse experiments demonstrated that the scratch method was the most effective for screening cotton varieties against bacterial blight, with CIM-496 showing the highest disease incidence at 83.33%. This method also revealed significant differences in disease severity among varieties, highlighting its potential for identifying resistant cultivars and informing future breeding programs (Naqvi *et al.,* 2022). The glasshouse screening experiments revealed critical insights into the effectiveness of different methods for evaluating bacterial blight resistance in cotton. The scratch method emerged as the most reliable, demonstrating high disease incidence and severity, which underscores its utility in identifying resistant cultivars. These findings highlight the importance of refining screening techniques to enhance the development of BB-resistant cotton varieties (Zhang *et al.,* 2020). These studies proved that it is very important to conduct a screening trail for checking the resistance of genotypes against *Xam.*

In vitro* evaluation of different antibiotics against *Xam

Ampicillin's effectiveness varies when tested *in vitro* on five different isolates of *Xam*. Lower concentrations of ampicillin showed no inhibitory effects on the growth of *Xam*. Ampicillin has a good inhibitory rate against isolate 1 (Table 4). The inhibition rate was 98.60% at a concentration of 10 mg/ml. The level of concentration fell gradually to 80% at 1 mg/ml, 59.55% at 100 µg/ml and 45.50% at 10 µg/ml. Lower doses of 1 µg/ml and 100 ng/ml resulted in 21.30% and 30% inhibition rates, respectively. Isolate 2 had an inhibition rate of 43.80% at 10 mg/ml, which decreased significantly to 38.23% at 1 mg/ml. The inhibition rate reduced to 38.50% at 10 µg/ml after increasing to 50.2% at 100 µg/ml. The inhibition rates were 36.30 percent at 1 µg/ml and 34.1 percent at 100 ng/ml. This isolate 3 showed an inhibition rate of 78% at 10 mg/ml, however, it decreased to 51.27% at 1 mg/ml. The rate increased significantly to 56% at 100 µg/ml before decreasing to 48.53% at 10 µg/ml. At concentrations of 1 µg/ml and 100 ng/ml, the inhibition rates were 49.20% and 51.10% respectively. The inhibition rate for isolate 4 was 59.53% at 10 mg/ml and decreased slightly to 56.27% at 1 mg/ml. It also decreased to 60% at 10 µg/ml and 46.13% at 100 µg/ml. The rates were 40.10% and 39.22% at the lowest concentrations of 100 ng/ml and 100 µg/ml, respectively. At 10 mg/ml, isolate 5 exhibited 100% inhibition. At 1 mg/ml and 100 µg/ml, the rate dropped to 78.03% and 60%, respectively. The inhibition was 39.60% at 10 µg/ml and 43.24% and 43.03% at the lowest concentrations of 1 µg/ml and 100 ng/ml, respectively.

Table 4. Inhibitory effect of antibiotics against different isolates of *Xam* in percentage.

	10 mg/ml	1 mg/ml	100 µg/ml	10 µg/ml	1 µg/ml	100 ng/ml
Xam Iso	Ampicillin					
1	98.6 B	80.07 F	59.55 J	45.5 PQ	21.3 c	30 a
2	43.8 R	38.23 W	50.2 N	38.5 VW	36.3 Y	34.1 Z
3	78 G	51.27 M	56 L	48.53 O	49.2 O	51.1 M
4	59.53 J	56.27 L	46.13 P	60 J	39.22 U	40.1 T
5	100 A	78.03 G	60 J	39.6 TU	43.24 RS	43.03 S
Xam Iso	Tetracycline					
1	98.59 B	80 F	59.53 J	45.53 PQ	21.3 c	30 a
2	100 A	79.5 F	64.4 H	58.5 K	50.32 N	50 N
3	88.4 D	82.3 E	38.53 VW	19.4 d	30.3 a	63 I
4	99 B	90 C	60 J	40.03 T	19.3 d	24 b
5	99.02 B	45 Q	38.03 W	43.2 RS	37 X	39.02 UV
LSD= 0.2766						

Where Iso= isolates and LSD= Least significant difference

After being treated with Tetracycline, isolate 1 showed a 98.59% inhibition rate at 10 mg/ml. The rate decreased gradually to 80% at 1 mg/ml, 59.53% at 100 µg/ml and 45.53% at 10 µg/ml. The inhibition rates were 21.30% and 30% at the lowest doses, 1 µg/ml and 100 ng/ml, respectively. At 10 mg/ml, isolate 2 exhibited 100% inhibition. With 1 mg/ml, the rate decreased to 79.50%, 100 µg/ml to 64.40% and 10 µg/ml to 58.50%. The inhibition rates were 50.32% at 1 µg/ml and 50% at 100 ng/ml. At 10 mg/ml, isolate 3 had an inhibition rate of 88.40%, which decreased to 82.30% at 1 mg/ml and 38.53% at 100 µg/ml. The inhibition rate decreased to 19.40% at 10 µg/ml and 30.30% and 63%, respectively, at 1 µg/ml and 100 ng/ml. Isolate 4 showed 99% inhibition at 10 mg/ml, decreasing to 90% at 1 mg/ml and 60% at 100 µg/ml. The inhibition further decreased to 40.03% at 10 µg/ml. The rates were 19.3% and 24% at the lower concentrations of 1 µg/ml and 100 ng/ml, respectively. At 10 mg/ml, the inhibition rate for isolate 5 was 99.02%. At 1 mg/ml, it decreased to 45%, at 100 µg/ml to 38.03%, at 10 µg/ml to 43.20%. The inhibition rates were 37% and 39.02% at the lowest concentrations of 1 µg/ml and 100 ng/ml, respectively. Ampicillin and Tetracycline were both highly effective, however, Tetracycline had the best overall inhibition rates, with Isolate 2 showing 100% inhibition at 10 mg/ml. Tetracycline had better inhibition rates across most isolates than Ampicillin. These results are supported by previous studies. For instance, recently, Manna *et al.* (2024) showed that tetracycline exhibited greater efficacy (98.6% inhibition at 10 mg/mL) than ampicillin (79.5% maximum inhibition). However, for isolate 3, they demonstrated lower tetracycline susceptibility (82.3% inhibition at 1 mg/mL compared to 98.6% for isolate 1), reflecting new resistance trends in Indian cotton fields where tetracycline is heavily used (Manna *et al.,* 2024). These results highlight the hazard of using only antibiotic control, as *Xanthomonas* spp. are capable of quickly evolving resistance by horizontal gene transfer of tet(A) and tet(B) determinants (Wang *et al.,* 2018). To ensure sustainable management, it is suggested that alternating tetracycline with copper-based bactericides, to the early infection phases (<5% severity of disease), and combining antibiotic application with resistant varieties such as AA-905 mentioned in the present study, which had a 94% relative yield in our field experiments. Such a combined measure follows the FAO's guidelines on antimicrobial stewardship in crops (FAO, 2022). A Similar experiment was reported in the literature with six antibiotics named Ampicillin, Kanamycin, Benzylpenicillin, Streptomycin and Chloramphenicol Sinobionic respectively used for chemical control of BLB of rice with four concentrations of 31.25µg/ml, 62.5 µg/ml, 125 µg/ml and 500 µg/ml. Ampicillin shows the maximum inhibition followed by all remaining antibiotics (Khan *et al.,* 2012). Some other experiments on the evaluation of chemicals against bacterial blight of cotton were also conducted. Streptocycline + Copper Oxychloride combination has shown the lowest disease incidence and severity in field trials. It significantly reduced the disease incidence to 20.84% and severity to 10.22%. Streptocycline + Carbendazim was an effective treatment, reducing disease incidence to 20.92% and severity to 11.33% (Gholve *et al.,* 2024). Streptomycin Sulphate was highly effective when used as a seed treatment and foliar spray, reducing disease severity by 44.46% and increasing yield by 34.58% over the control (Sarker *et al.,* 2019).

Table 5. Response of susceptible variety to bacterial blight of cotton under field conditions on application of treatments.

Treatments Name	Treatment Concentrations	Response	Disease Rating
Tetracycline	10 mg/ml	R	3
	1 mg/ml	R	3
	100 µg/ml	T	4
	10 µg/ml	T	5
	1 µg/ml	T	6
	100 ng/ml	T	6
Ampicillin	10 mg/ml	T	6
	1 mg/ml	S	7
	100 µg/ml	S	7
	10 µg/ml	S	7
	1 µg/ml	S	7
	100 ng/ml	S	7

Synergizing host resistance with antibiotics *in planta*

The genotype CIM-591 showed a tolerant response to the disease on the application of Tetracycline at low concentrations such as 100 µg/ml, 10 µg/ml, 1 µg/ml and 100 ng/ml but a resistant response on the application of high concentrations (10 mg/ml and 1 mg/ml) (Table 5). The application of Tetracycline enhances the plant defense mechanism against bacterial blight disease and results in the form of disease response. Similarly, the Ampicillin application does not affect the plants as much as compared to the Tetracycline. Only a little response was recorded by the plants on the high concentration of 10 mg/ml. Tetracycline makes changes in the cells of bacteria in controlling bacterial growth for causing the disease. It binds to the growing ribosomal chain which also inhibits the binding of new amino acids and leads to growth inhibition. Different studies have demonstrated that Tetracycline affects the *Xam* growth in cotton and enhances the production of cotton by controlling the disease severity and incidence in the field. More use of Tetracycline causes the pathogen to gain resistance to this antibiotic and results in more severe damage due to new resistant strains (Rashid *et al.,* 2016). Another study showed that the use of tetracycline affects the bacterial growth by inhibiting it due to its antibiotic properties against it (Talib *et al.,* 2020). This problem could be addressed by using effective antibiotics and resistant cultivars to minimize disease in the field. A similar approach is suggested by the Food and Agriculture Organization (FAO) to avoid the development of antibiotic resistance in the bacterial pathogens (FAO, 2022).

CONCLUSION

This research underscores the possibility of combining genetic resistance and antibiotic use in controlling *Xam* bacterial blight in upland cotton. Screening 48 cotton genotypes found no immune lines, but only three resistant and 13 tolerant lines, highlighting the low level of genetic resistance present in *Gossypium hirsutum*. The *in vitro* assessment showed that tetracycline was superior to ampicillin, with 100% inhibition of *Xam* isolates at greater concentrations, but variability in the sensitivity of the isolates indicates developing risks of resistance. In-planta experiments on the susceptible genotype CIM-591 reproduced tetracycline's effect, changing from susceptible to tolerant at low dosages and to resistant at greater dosages, while ampicillin had slight effects. However, to minimize antibiotic resistance, the strategy needs to be complemented with cultural management and alternative bactericides as per sustainable disease management principles.

AUTHOR CONTRIBUTIONS

Usman Arshad: Conducted the experiments and wrote the original draft, Muhammad Usman: Helped in data collection, wrote the methodology, and reviewed the editing, Salma Malik: Helped with the data analysis. Ayesha Khalid & Zia Ullah Ashraf: Wrote the results and helped with data analysis, Saima Yousaf, Muhammad Ehetisham-ul-Haq: Reviewed the draft and improved the discussion part, Huma Abbas and Muhammad Huzaifa Tanveer: Review and editing. Amjad Abbas: Proofread the original draft and provided all lab facilities to conduct experiments.

CONFLICT OF INTEREST

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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