

Isolation and Characterization of Three Pathogenic Strains of *Xanthomonas* from Santiniketan, West Bengal Causing Diseases in Economically Important Crops

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Abstract

Xanthomonas represents a large genus of gammaproteobacteria that comprises several plant pathogenic strains causing significant plant diseases in various economically important crop plants. The Santiniketan region of West Bengal is considered as a Red and Lateritic agro-climatic zone, where bacterial leaf blight diseases of rice, black rot of cruciferous crop plants, and citrus canker have emerged as significant bacterial plant diseases caused by different strains of *Xanthomonas*. The present study demonstrated the isolation and characterization of different pathogenic strains associated with these plant diseases. Among a number of isolates, three were selected and characterized based on their colony morphology, disease development efficiency, and various virulence-associated traits, including flagellar motility, cellulase production, biofilm formation, and siderophore synthesis.

Keywords: Plant pathogenic bacteria, *Xanthomonas*, Virulence-associated function

1. Introduction:

Plant disease outbreaks have emerged as an unavoidable cause of a 10% loss in global food production, leading to a huge agro-economic loss of approximately \$ 400 billion every year worldwide [1]. Among various microbial phytopathogens, plant pathogenic bacteria form a large and diverse group of pathogens that cause diseases in a wide range of agronomically important plants worldwide. However, the genus *Xanthomonas* comprises more than 35 species and their associated pathovars, which cause diseases in over 400 host plants, including approximately 124 monocotyledonous and 268 dicotyledonous plants [2]. It is a rod-shaped, aerobic, Gram-negative bacterium that has a polar flagellum and usually forms round, convex, mucoid, yellow colonies, as it harbors a characteristic yellow pigment, Xanthomonadin [3]. *Xanthomonas* is usually transmitted through infected seeds or plant debris, or dispersed through wind or rain splashes, resulting in infection and epiphytic colonization. Later, bacterial cells enter the plant tissue through natural openings, such as stomata, hydathodes, or other openings in the leaves. After invading the plant tissue, the bacteria initiate infection and colonization in specific tissues, including vascular bundles and leaf mesophyll, depending on their tissue specificity [3,4,5]. Different pathovars of *Xanthomonas* have been identified as associated with various symptoms and diseases. Successful invasion, colonization, and disease development by different pathovars of *Xanthomonas* are supported by multiple environmental

parameters and various endogenous factors, including the synthesis of extracellular polysaccharides, the production of diverse virulence factors, the production of siderophores, and quorum sensing. *Xanthomonas* infection is initially restricted to localized disease development, which often results in systemic infection and the death of the entire host plant [3,4,6]. The diseased host plant can function as a source of horizontal transmission throughout the entire crop field, ultimately resulting in significant agro-economic losses.

A part of the Birbhum district of West Bengal, including Santiniketan has been considered as an important part of the Red and Lateritic agroclimatic zone [7]. Bacterial blight of rice, black rot of cruciferous plants, and canker of citrus have emerged as the most common plant diseases caused by different pathovars of *Xanthomonas*. Different pathovars or strains causing these plant diseases in this agroclimatic zone have been studied little so far. The present work demonstrated the isolation and characterization of the causal organisms of these diseases in Santiniketan, which represents the *Xanthomonas* strains associated with these diseases in this Red and Lateritic agroclimatic zone.

2. Materials and methods:

Materials: Bacterial growth media and bactoagar were procured from HiMedia Laboratories. Different reagents and salts were purchased from Sisco Research Laboratories (SRL).

Isolation of pathogenic strains of *Xanthomonas*: Infected plant samples (rice leaves with bacterial leaf blight, cabbage leaves with black rot disease, and citrus leaves with prominent canker) were collected from various localities in Santiniketan, Birbhum, West Bengal. Diseased leaves were appropriately washed with sterile water and then surface sterilized with 0.1% HgCl_2 , followed by a transverse cut at the diseased area with sterile scissor and placed on semi-selective medium, YPGA (yeast extract, bacto peptone, glucose, bactoagar) [8,9], supplemented with cycloheximide 50 mg/ml for proper oozing of the pathogenic bacteria from the diseased parts. The plates were kept in a static incubator at 28°C for 2-3 days, without inverting the petri dishes. The yellow, glossy bacterial colonies were picked up and subjected to dilution streaking to obtain a pure culture of the isolates. Six isolates from each diseased plant sample were maintained in PSA (bacto peptone, sucrose, bactoagar) plates as pure cultures and stored as 20% glycerol stocks in a -80°C deep freezer [5,6].

In planta assay: Six isolates isolated from diseased rice leaves were grown in PS (bacto peptone, sucrose) broth at 28°C at 200 rpm shaking up to mid-log phase, and in planta infection was performed by clip inoculation method in healthy 45 days old rice leaves, and rice plants were maintained in the greenhouse [5]. Similarly, 30-day-old healthy cabbage leaves were infected by the clip inoculation method using a sterile scissor. Six isolates were isolated from diseased cabbage leaves, grown to the mid-log phase in PS broth at 28°C with 200 rpm shaking, and cabbage plants were maintained in a greenhouse [6]. Likewise, six isolates isolated from diseased citrus leaves were grown in PS broth at 28°C with 200 rpm shaking until the mid-log phase, and in planta infection was performed by the infiltration method in healthy 60-day-old citrus leaves. Citrus plants were maintained in a greenhouse [5].

External and microscopic study: Selected pathogenic isolates were maintained as pure cultures, and colony morphologies were observed. Additionally, microscopic observation was performed using a light microscope on mid-log phase liquid cultures of the selected isolates after Gram staining.

Growth assay: Growth assays of different *Xanthomonas* strains were performed following a standard protocol [5,6]. Selected isolates were grown to mid-log phase in PS, centrifuged, and washed with sterile

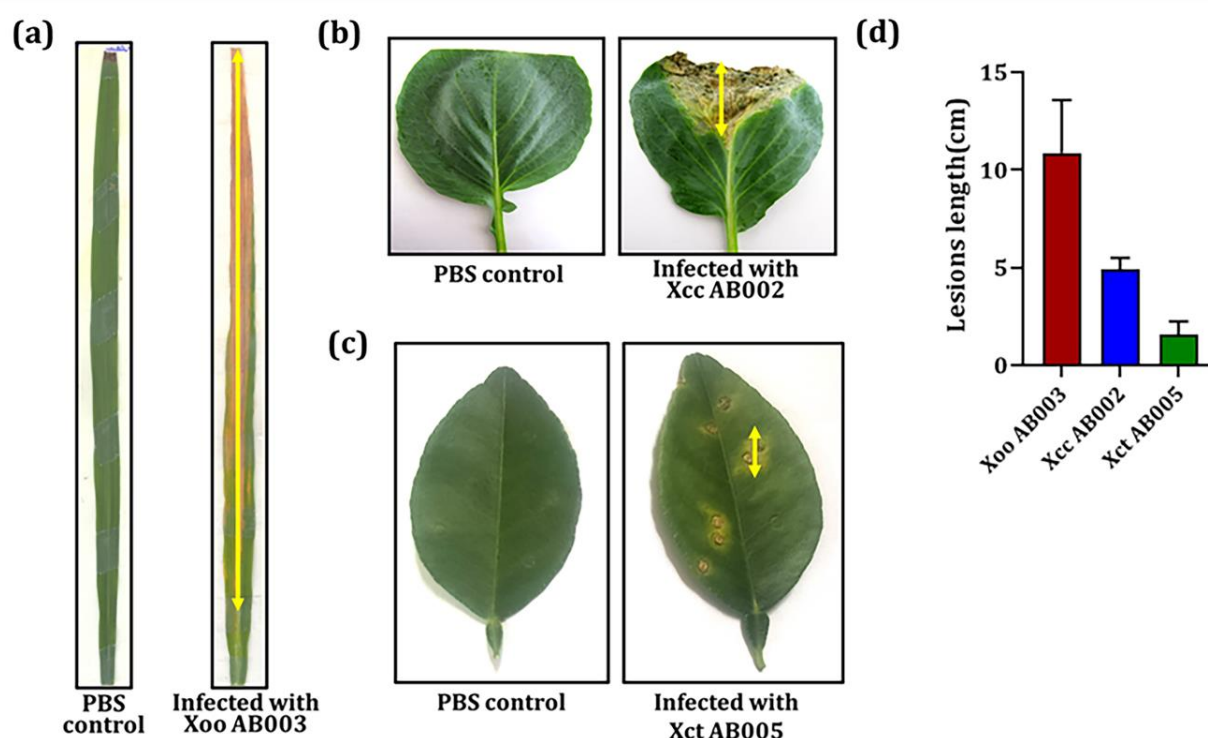
PBS. These cultures were then normalized to an OD600 of 0.5, and 0.1% of the normalized cultures were grown in PS medium for up to 60 h at 28°C and 200 rpm in a shaker incubator (Researchers' Pal, India).

Assays for virulence-associated traits: Assays for virulence-associated traits, such as swimming motility, cellulase production, biofilm formation, and siderophore synthesis, were performed using a standard protocol [5,6].

3. Results and discussion:

The genus *Xanthomonas* comprises a diverse array of plant pathogenic bacterial species that cause significant diseases in economically important crops. However, the most common diseases caused by *Xanthomonas* are the bacterial leaf blight of rice caused by *X. oryzae* pv. *oryzae*, black rot disease in cruciferous crops, including mustard, cabbage, cauliflower, etc., caused by *X. campestris* pv. *campestris*, and citrus canker caused by *X. citri* pv. *citri* [5,6,10].

Diseased plant parts were examined and then collected carefully from agricultural fields and gardens in different areas of Santiniketan. Bacterial leaf blight of rice was observed in the agricultural field at Goyalpara, Santiniketan, West Bengal, and diseased leaf samples were collected following standard protocol [8,9]. Likewise, black rot disease in cabbage was observed in the agricultural field at Ballavpur, Santiniketan, West Bengal, and diseased leaf samples were collected. Similarly, Leaf canker of citrus was observed in a garden at Purbapally, Santiniketan, West Bengal, and diseased leaf samples were collected. The diseased leaf samples were washed with sterile water, surface sterilized by HgCl₂, followed by cutting open the diseased areas of the leaf sample with a sterile scissor and kept on a semi-selective agar media (supplemented with cycloheximide 50 µg/ml) so that the ooze can out from the diseased leaf samples [9]. After incubation at 28°C for 2-3 days, bacterial colonies were developed on the agar plates. The glossy, yellow colonies were picked up and made pure culture by dilution streaking. Single colonies were picked and maintained in -80°C deep freezer for prolonged storage. A single bacterial colony from the isolates of



each pathogenic strain was further maintained in PSA media for regular experimental purposes [5,6]. Colony morphology and Gram staining of all the isolates were observed (Table 1).

Figure 1: (a-c) In planta virulence assay and development of disease symptoms by three pathogenic isolates of *Xanthomonas* (Xoo AB003, Xcc AB002, Xct AB005, respectively). (d) Comparative study of the lesion developed by the isolates, Xoo AB003, Xcc AB002, Xct AB005.

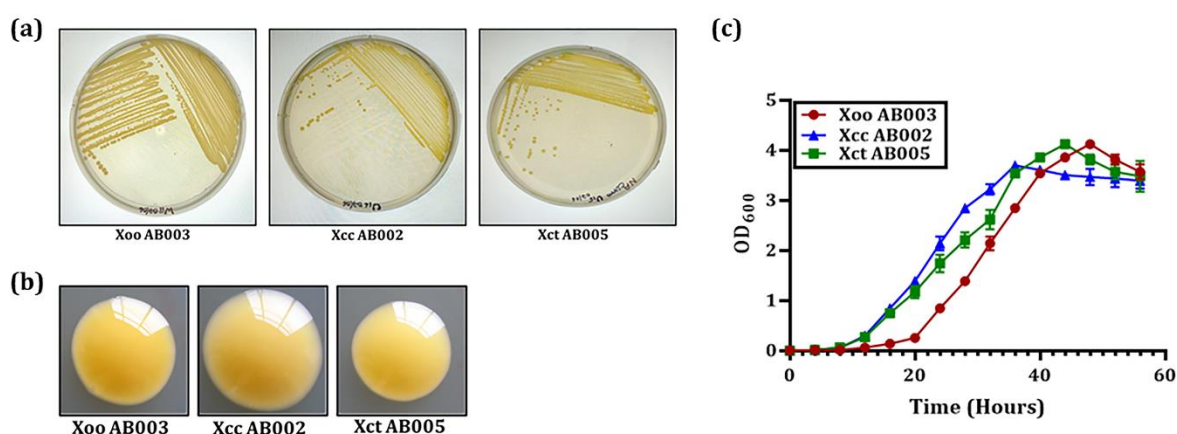
Table 1: Comparative account of the virulence and morphological characterization of the isolates

Diseased plant samples used	Isolates	Colony morphology	Gram property	Lesion length (cm)
Rice leaves having bacterial leaf blight	Xoo AB001	Round, Yellow, Convex	Gram (-ve)	9.3
	Xoo AB002	Round, Yellow, Convex, Mucoid	Gram (-ve)	11.6
	Xoo AB003	Round, Yellow, Convex, Mucoid	Gram (-ve)	13.7
	Xoo AB004	Round, Yellow, Convex, Mucoid	Gram (-ve)	10.5
	Xoo AB005	Round, Yellow, Mucoid	Gram (-ve)	No Lesion
	Xoo AB006	Round, Yellow, Convex, Mucoid	Gram (-ve)	11.9
Cabbage leaves having black rot	Xcc AB001	Round, Yellow, Convex, Mucoid	Gram (-ve)	4.8
	Xcc AB002	Round, Yellow, Convex, Mucoid	Gram (-ve)	5.3
	Xcc AB003	Round, Yellow, Mucoid	Gram (-ve)	3.7
	Xcc AB004	Round, Yellow, Convex, Mucoid	Gram (-ve)	4.1
	Xcc AB005	Round, Yellow, Convex, Mucoid	Gram (-ve)	5.1
	Xcc AB006	Round, Yellow, Convex	Gram (-ve)	No Lesion
Citrus leaves with canker	Xct AB001	Round, Yellow, Convex, Mucoid	Gram (-ve)	1.6
	Xct AB002	Round, Yellow, Convex, Mucoid	Gram (-ve)	2.0
	Xct AB003	Round, Yellow, Convex, Mucoid	Gram (-ve)	1.3

	Xct AB004	Round, Muroid	Yellow,	Convex,	Gram (-ve)	1.8
	Xct AB005	Round, Muroid	Yellow,	Convex,	Gram (-ve)	2.3
	Xct AB006	Round, Muroid	Yellow,	Convex,	Gram (-ve)	1.7

Six isolates from each diseased plant sample were applied to healthy host plants. Healthy rice leaves (45 days old) and healthy cabbage leaves (30 days old) were inoculated with mid log culture of *X. oryzae* pv. *oryzae* (Xoo) and *X. campestris* pv. *campestris* (Xcc) isolates respectively, following clip inoculation method [6]. However, healthy citrus leaves were infected with *X. citri* pv. *citri* (Xct) following infiltration method. Among the six isolates from each pathogenic strain, Xoo AB003, Xcc AB002, and Xct AB005 were selected based on their efficiency in disease development (Table 1). *X. oryzae* pv. *oryzae* (strain Xoo AB003) caused yellowish, water-soaked, long lesions from the tip of the rice leaves through the rice leaf mid vein (about 10 cm long) after 15 days post inoculation (Figure 1a). *X. campestris* pv. *campestris* (strain Xcc AB002) caused a yellowish, V-shaped, water-soaked necrotic region (about 5 cm long), similar to black rot disease of cruciferous plants, after 15 days post-inoculation (Figure 1b). *X. citri* pv. *citri* (Xct AB005) caused brownish pustules along with yellowish spots on the citrus leaf (about 2 cm in diameter), after 30 days post-inoculation (Figure 1c). The disease symptoms developed during the in planta virulence assay, using the isolates, were similar to those from which they were isolated. However, the pathogens isolated from the experimental set were identical to that of the primary isolates.

All the selected isolates, Xoo AB003, Xcc AB002, Xct AB005 exhibited similar colony morphology. The colonies were round, convex, glossy, mucoid, and yellow in colour (Figure 2a & 2b). The yellow appearance is a signature of any *Xanthomonas* species as it bears a yellow pigment named as Xanthomonadin [3]. The glossy and mucoid appearance is mainly due to significant production of extracellular polysaccharides (EPS) by the isolates [3]. Light microscopic observation after Gram staining revealed that all the strains are Gram-negative in nature, rod-shaped, but randomly arranged, rather than forming any chain or grape-like aggregate (data not shown). All three strains, Xoo AB003, Xcc AB002, and Xct AB005, differ little in their growth patterns in peptone sucrose (rich medium) broth (Figure 2c).



However, Xcc AB002 and Xct AB005 exhibited faster growth rate (generation time of 2.2 hrs and 2.5 hrs) compared to Xoo AB003 (generation time of 4 hrs).

Figure 2: (a) Three pathogenic isolates (Xoo AB003, Xcc AB002, Xct AB005, respectively) after dilution streaking for getting pure culture. (b) Colony morphology of the isolates. (c) Comparative growth kinetics of the isolates in PS broth (Xoo AB003, Xcc AB002, Xct AB005).

However, all three strains were checked for their virulence-associated traits, including flagellar motility, cellulase synthesis, biofilm formation, and siderophore synthesis (Table 2). All of the three strains, Xoo AB003, Xcc AB002, Xct AB005 exhibited significant swimming motility (Figure 3a) in motility semi-solid plates containing 0.7% agar [5,6]. However, Xoo AB003 exhibited more flagellar motility compared to Xcc AB002 and Xct AB005. Flagellar motility enables pathogenic bacteria to engage in flagella-driven chemotaxis, facilitating successful invasion and colonization within host tissue [3]. Cellulase synthesis was demonstrated on Carboxymethyl cellulose (CMC) agar plates, and all three strains, Xoo AB003, Xcc AB002, and Xct AB005, showed significant cellulase synthesis (Figure 3b). Cellulase production is one of the essential virulence traits of *Xanthomonas*, as it is secreted through the Type-II secretion system (T2SS), which plays a vital role in pathogenesis by secreting essential enzymes and virulence factors involved in its pathogenesis [5]. Similarly, biofilm formation has been considered one of the essential virulence-associated functions of most pathogenic bacteria, as it facilitates surface attachment and colonization, leading to disease development. Additionally, biofilm formation helps *Xanthomonas* in combating various hostile environmental conditions and stresses [3]. All three strains, Xoo AB003, Xcc AB002, Xct AB005 exhibited notable and similar biofilm formation on a solid surface (Figure 3c). Interestingly, all three strains, Xoo AB003, Xcc AB002, Xct AB005 exhibited remarkable siderophore (Xanthoferrin) production under iron-limiting conditions (Figure 3d), when assayed on peptone sucrose agar (PSA)- chrome azurol sulfonate (CAS) siderophore indicator plates containing 75 μ M of the ferrous ion chelator dipyrrolyl (DP). *Xanthomonas* considers an external iron-starving condition as a signal for initiating its virulence function, and it co-regulates iron homeostasis and virulence-associated functions in a coordinated manner. So, siderophore production can be considered as an essential virulence-associated trait of *xanthomonads* [3,5,6].

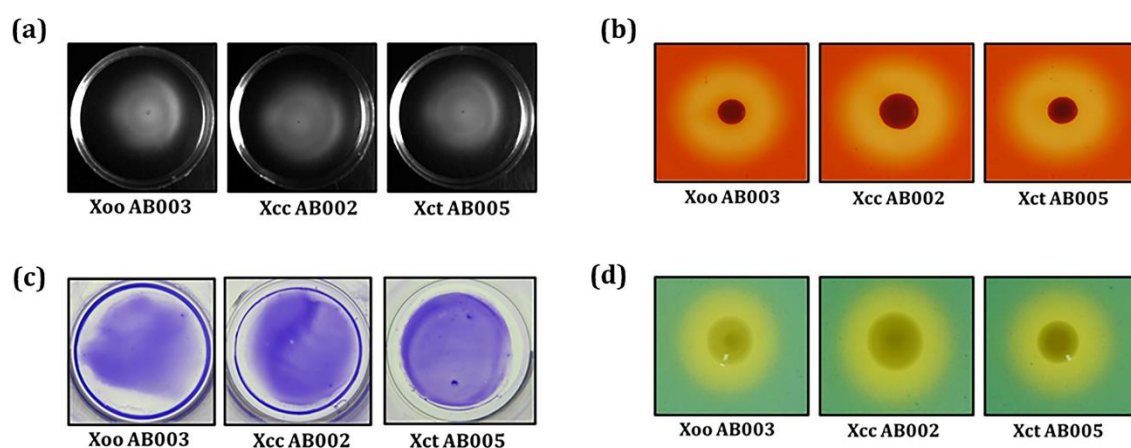


Figure 3: (a) Swimming motility plates showing flagellar motility of three isolates. (b) Cellulase production by three isolates on CMC plates. (c) Biofilm formation efficiency of three isolates. (d) Siderophore production on PSA-CAS plates containing 75 µM DP (iron chelator).

Table 2: Comparative account of the essential virulence-associated traits of the three pathogenic strains of *Xanthomonas* isolates

Pathogenic strains of <i>Xanthomonas</i> sp.	Isolates	Flagellar motility (Motility Zone diameter)	Cellulase production (Hallow zone diameter)	Biofilm formation (Absorbance at 570 nm)	Siderophore production (Hallow zone diameter)
<i>X. oryzae</i> pv. <i>oryzae</i>	Xoo AB003	4.2±0.98 cm	0.8±0.66 cm	1.7±1.3	0.5±1.6 cm
<i>X. campestris</i> pv. <i>campestris</i>	Xcc AB002	3.5±0.57 cm	0.7±0.29 cm	2.0±0.85	0.7±0.78 cm
<i>X. citri</i> pv. <i>citri</i>	Xct AB005	3.2±1.2 cm	0.8±0.71 cm	1.5±1.3	0.4±1.4 cm

4. Conclusion:

Different plant pathogenic strains like *X. oryzae* pv. *oryzae*, *X. campestris* pv. *campestris*, and *X. citri* pv. *citri* of *Xanthomonas* were isolated from the diseased rice leaves, cabbage leaves, and citrus leaves, respectively. Out of six isolates from each diseased sample, one strain was selected from each pathogenic strain based on their virulence efficiency. Further investigation revealed that these strains exhibit more or less similar colony morphology and Gram staining properties, but they differ in their growth kinetics and essential virulence-associated traits, such as flagellar motility, cellulase production, biofilm formation, and siderophore synthesis. These strains may be associated with the concerned plant diseases throughout the Red and Lateritic agro-climatic zones of West Bengal.

5. **Conflict of interest:** The author declares no conflict of interest.
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