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Isolation, biochemical characterization and pathogenicity of *Xanthomonas campestris* pv. *campestris*

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Abstract
Cabbage is one of the important cole crop grown around the world. Cabbage is susceptible to numerous diseases. However, the most destructive among them is black rot. Pathogen was isolated from leaves showing typical V- shaped lesions and identified as *Xanthomonas campestris* pv. *campestris* through morphological and biochemical characteristics. The biochemical tests showed that *Xcc* is small rod shaped, gram negative in gram staining and gave positive result for KOH test, starch hydrolysis test and negative for oxidase test. Pathogenicity of *Xcc* was confirmed according to Kotch's postulate.

Keywords: Black rot, biochemical test, *Brassica oleracea* var. *capitata*, pathogenicity test

Introduction
Cabbage (*Brassica oleracea* var. *capitata*) is an important cole crop belongs to the cruciferae family. Cabbage holds immense horticultural importance in India it is cultivated as a crucial vegetable crop. It is known for its antibacterial and antiviral properties. Cabbage is rich source of vitamin-C, carbohydrates, dietary fibre, proteins and trace amounts of vitamins and minerals.

Cabbage is susceptible to numerous diseases, including powdery mildew, downy mildew, clubroot, root rot and damping-off. However, the most destructive among them is black rot, it is caused by the seed-borne bacterium *Xanthomonas campestris* pv. *campestris* (*Xcc*) (Pammel, 1895) [6] which is prevalent in tropical and subtropical regions of the world. This pathogen is responsible for causing significant economic losses on a global scale. Initial symptoms of the black rot disease include water soaked distinct yellow 'V'-shaped areas along the leaf margins, with the internal tissue transitioning from brown to black.

Biochemical tests were conducted for characterization of *Xcc*. Gram staining, catalase test, oxidase test, KOH test and starch hydrolysis (Patidar and Ranjan, 2022) [7]. *Xcc* is a bacterium characterized by its small, straight rod shape, and measuring 0.5 - 0.75 mm in width and 1.5 - 2.0 mm in length. It is mobile, aerobic, gram-negative, non-spore-forming and an obligate bacterium. This pathogen possesses a single polar flagellum and exhibits catalase-positive, hydrogen sulfide-positive, oxidase-negative traits, while it does not produce nitrate or indole. *Xcc* demonstrates a broad temperature tolerance range for survival, spanning from 5 - 38 °C, with an optimal growth temperature at 30 °C, but it becomes lethal at around 50 °C (Sidhu *et al*., 2008) [9]. Proving pathogenicity of *Xcc* on cabbage plant was carried out by following standard protocol developed by Chitarra *et al*. (2002) [2].

Material and Methods
Symptomatology
Black rot affected leaves of cabbage plant were collected for isolation and identification of the pathogen. The symptoms were studied and recorded.

Isolation of *Xanthomonas campestris* pv. *campestris*

Cabbage leaves showing distinct V-shaped lesions. The infected leaves were collected and pathogen was isolated. After a thorough wash, the affected regions were cut into small fragments and then surface-sterilized using 70 percent ethyl alcohol for one minute. Subsequently, the fragments underwent three rinses with water and were crushed on a sterilized glass slide to create a bacterial suspension. To achieve individual, isolated bacterial colonies, this suspension was streaked onto petri plates containing nutrient agar medium. These plates were then incubated at 32 °C for 48 hours. On the third day of incubation, pure, shining yellow droplet-like colonies were observed on the petri plates.

Purification of *Xcc*

The bacterial colonies, which exhibited the characteristic yellow, slimy and glistening traits of *Xcc*, were individually purified by streaking them onto nutrient agar medium. Using a sterilized inoculation loop, these colonies were meticulously transferred to the surface of sterilized petri dishes containing nutrient agar medium. For long-term preservation, the bacterium was stored at -80 °C in glycerol solution (50% w/v), as described by (Kiran Kumar, 2007) [3].

Biochemical characterization of *Xcc*

Biochemical tests were conducted for characterization of *Xcc*. Gram staining, catalase test, oxidase test, KOH test and starch hydrolysis (Patidar and Ranjan, 2022) [7].

Gram's reaction: Test culture was placed on clean slide then heat fix it and added one drop of crystal violet stain then allowed for two minutes then washed with tap water and air dried for two seconds. Then gently flood with Gram's iodine and allowed for 1 minute. Then again washed in tap water and air dried. Then added 95% ethyl alcohol decolorizing agent and allowed for two minutes. Then added secondary stain, safranin, allowed for one minute then wash and air dried. Then slide was observed under microscope for gram reaction.

Catalase test

To assess catalase enzyme production, a clean microscopic slide was employed. Aseptically, a drop of 3% H_2O_2 was placed on the microscopic slide. A loopful of culture was then combined with the 3% H_2O_2 solution on the slide. Observations were recorded based on the presence or absence of bubble production.

Oxidase test

A filter paper disk was saturated with the substrate tetramethyl-p-phenylenediamine dihydrochloride. An inoculation loop was used to apply a small amount of culture to the paper. Observations were recorded based on color change of the paper to a deep blue or purple within a 10-30 second timeframe.

KOH test

The potassium hydroxide string test is a quick method to differentiate whether bacteria are Gram-positive or Gram-negative. Using a micropipette, a drop of KOH (3%) was placed on a clean slide and with help of cooled sterilized wire loop, a portion of a single colony of *Xcc* (48 h old culture) was isolated from NSA medium and blended on the slide with KOH solution by inoculating loop. Slimy thread was formed when inoculating loop was move up gently.

Starch hydrolysis test: The autolaved and cooled starch agar medium was poured in sterile glass Petri plate and on solidification of the medium, streaked pure culture of the test bacterium and incubated for 96 hrs at 28 °C. Then these plates was flooded with lugol's iodine and allowed to react for few minutes. Reddish coloured zones indicate negative reaction and appearance of yellowish, clear zones around the bacterial growth indicates positive reaction.

Proving pathogenicity of *Xcc*

Proving pathogenicity of *Xcc* on cabbage plant was carried out by following standard protocol developed by Chitarra *et al.* (2002) [2].

To adhere to Koch's Postulates and confirm the pathogenicity of bacterium, one-month-old cabbage plants were used. These plants were grown in 30 cm diameter pots and were inoculated with a 48-hour-old bacterial culture. The inoculation process involved puncturing the major veins of the first two true leaves at 5-10 points using a sterile needle contaminated with bacterial cell suspension ($5x10^8$ cfu/ml) scraped directly from a culture of nutrient broth medium. The pots containing the inoculated plants were then placed in a poly house with controlled temperature and relative humidity for up to 12 hours. The presence of characteristic V-shaped yellow lesions with blackened veins, observed after 7 days and pathogen was re-isolated and brought back into a pure form following previously described procedures. This sequence of events affirms a positive response to the pathogenicity test.

Results

Symptomatology

Black rot disease manifests with necrotic lesions on cotyledons or young seedling leaves, resulting in blackening and eventual leaf drop. On mature plants, symptoms include yellowing or dead tissue along leaf edges, which later develop into "V"- shaped chlorotic lesions. Close examination of infected leaves and stems revealed blackened veins within the affected tissue. Additionally, the disease is characterized by head rot, wilting and eventual plant death during its advanced stages.

Isolation and purification of *Xcc*

The bacterium responsible for causing black rot was isolated from cabbage leaves displaying characteristic V-shaped lesions. These infected leaves were collected and placed in a clean polythene bag, then transported to the laboratory for pathogen isolation. The diseased leaves were crushed on a sterilized glass slide to obtain a bacterial suspension, which was then streaked onto nutrient agar medium. Subsequently, the plates were incubated at 32 °C for 48 hours. On the third day of incubation, pure glistening yellow droplet-like colonies were observed on the petri plates.

The isolated bacterium displaying the typical yellow, circular, medium to dark yellow, slimy, glistering and opaque colonies of *Xcc*, were purified individually by streaking them on nutrient agar medium. Using a sterilized inoculation loop, these colonies were carefully transferred onto the surface of nutrient agar medium in sterilized petri dishes. Later, the petri dishes were incubated at 32 °C for 48

hours. Subsequently, the bacterial colonies were streaked onto nutrient agar medium slants and incubated at room temperature before being stored at 4 °C in a refrigerator for future use. For long-term storage, the bacterium was preserved at 80 °C with 50 percent (w/v) glycerol.

Biochemical characterization of *Xcc*

The biochemical tests were carried out to identify pure culture of bacterial isolate through the characteristic of cell physiology. Biochemical tests performed in this study include gram's reaction, catalase, oxidase, KOH and starch hydrolysis test.

Gram's reaction: The gram reaction test of *Xcc* revealed that the bacterial cells are gram-negative. When observed under oil immersion magnification (100X), the bacterial cells appeared red in color, and they exhibited a rod-shaped morphology.

KOH test: Formation of slime thread or loop is an indication of being gram-negative because gram negative bacteria have relatively fragile cell walls, bounded by an outer membrane. This is readily disrupted by exposure to 3% KOH releasing the viscous DNA. But gram-positive bacteria by contrast possess a thicker, more rigid cell wall which resists the disruptive effect of KOH. The present study revealed that *Xcc* showed positive reaction to KOH test.

Starch hydrolysis: Results revealed that *Xcc* produced colorless zone around bacterial growth on starch agar medium flooded with Lugol's iodine and showed positive for starch hydrolysis test. *Xcc* hydrolyzed starch by exoenzyme amylase and broken down to dextrins, maltose, and glucose/alpha-amylase.

Oxidase test: In the oxidase test, the bacterial cultures were able to carry out the oxidation of colorless reagent, tetramethyl p-phenylenediamene dihydrochloride, and failed to produce desired colour. Therefore, a negative reaction was observed for oxidase test of *Xcc*.

Catalase test: The observation of bubbles indicates that the isolated bacteria exhibited catalase-positive activity, as they facilitated the decomposition of H_2O_2 to release oxygen. It is a well-known characteristic of *Xcc* to be catalase positive.

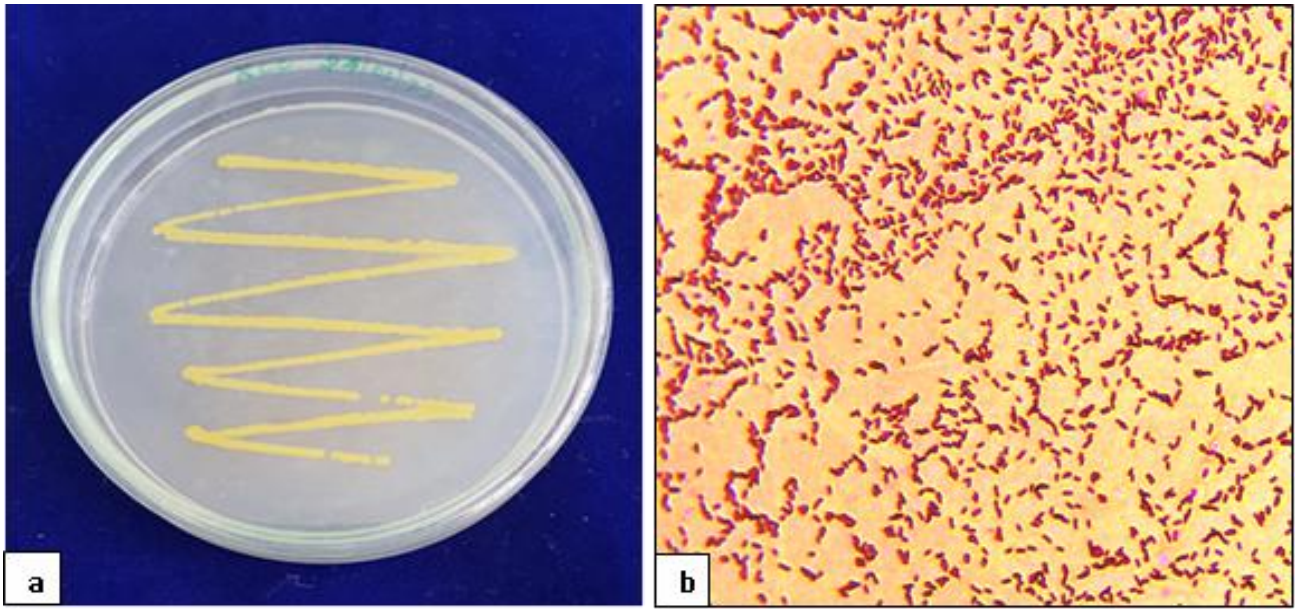
Table 1: Result of biochemical test for *Xcc*. '+' Positive test '_' Negative test

Sl. No.	Biochemical test	Result
1.	Gram reaction	-
2.	KOH test	+
3.	Starch hydrolysis	+
4.	Oxidase test	-
5.	Catalase test	+

Proving pathogenicity of *Xcc*

The pathogenicity of the bacterium was assessed by using standard protocol as detailed in the "Material and Methods" section.

Inoculated plants exhibited symptoms like V-shaped yellow lesions with blackened veins, closely resembling the ones observed in a natural environment. In contrast, the negative control, which was sprayed with distilled water, remained free of the disease. The bacteria was subsequently re-isolated from the affected plant parts, satisfying Koch's postulates, and maintaining morphological characteristics consistent with the originally isolated pathogen.



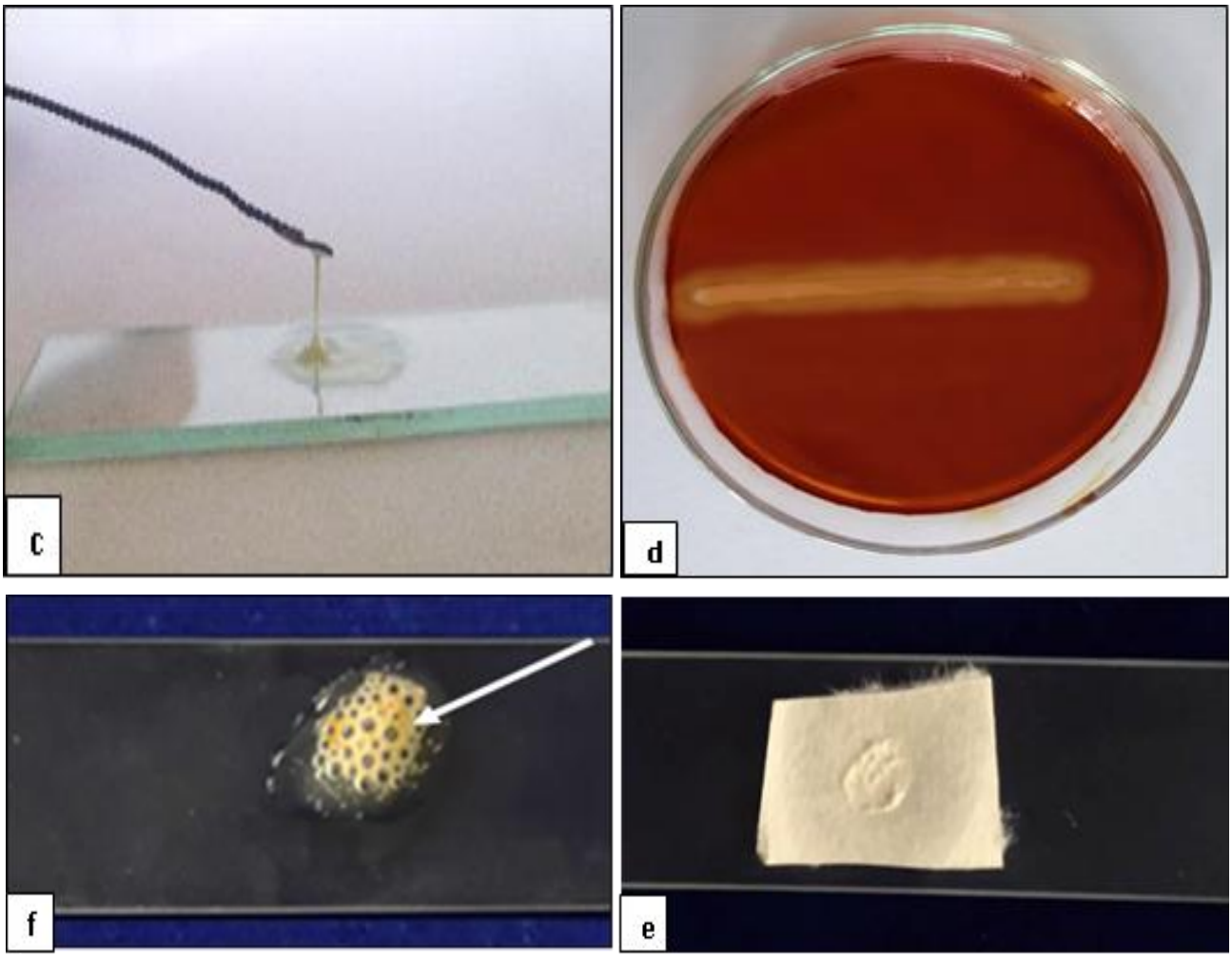


Fig 1: a) Pure culture of *X. c.* pv. *campestris,* b) Gram reaction, c) KOH test, d) Starch hydrolysis e) Oxidase test f) Catalase test

Discussion

Symptomatology

Initial symptoms of the disease appeared as chlorotic lesions along the margins of leaves which progress in the direction of midrib forming "V" shaped lesions. The veins and veinlets in the chlorotic area turned black and with the passage of time, the blackening of veins advanced to the stem. Advanced systemic infections caused darkened leaf veins and stem vascular tissue, extensive yellowing, wilting and necrosis of leaves. Similar symptomatological obsevations were also recorded by Vicente *et al*. (2001) [12] and Popovic *et al*. (2013) [8] in cabbage.

Isolation and purification of *Xcc*

The *Xcc* produce characteristic colonies of circular, medium to dark yellow, smooth flat, glistening, convex, round with fringed margin on nutrient agar medium after 48 hours of incubation (at 32 °C). Similar colonies of *Xcc* were also observed Vinayak (2012) [13].

Biochemical characterization of *Xcc*

On the basis of morphological, cultural and biochemical characteristics (Colour, size, margin, elevation, opacity) of the bacterium, it was identified as *Xcc.* And also bacterium was found positive for catalase reaction, starch hydrolysis, KOH and negative for oxidase test. Bacterial cells were rod shaped and gram-negative. Similar confirmations of *Xcc* were also recorded by Madhu (2009) [4] and Tanushree *et al*., (2020) [10].

Proving pathogenicity of *Xanthomonas campestris* pv. *campestris* (*Xcc*)

The pathogenicity was proved by inoculating bacterial cell suspension (5×10^8 cfu/ml) to the cabbage plants. The inoculated plants started expressing symptoms after 7 days of inoculation as similar symptoms was described in symptomatology and re-isolation of bacteria was observed as original culture of bacteria with respective colony characters. Zhao *et al*. (2000) [14] observed similar results where black rot isolates were capable of inducing disease symptoms when subjected to spray inoculation on certain plants within the crucifer family, serving as a pathogenicity test. Isolates from cruciferous hosts induced similar symptoms. Based on these observations, these isolates were identified as *Xcc* by Vicente (2013) [11], Maji and Nath (2015) [5] and Afrin *et al*. (2018) [1] also observed similar results.

Conclusion

Xanthomonas campestris pv. *campestris* causing cabbage black rot was isolated and biochemically characterized and proved pathogenicity. These findings contributes valuable insights to managing this pathogen, paving the way for further studies aimed at developing effective crop protection strategies.

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