



A study on pathological aspects of *Xanthomonas campestris* pv. *campestris* causing black rot of cabbage under red lateritic zone of West Bengal

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Abstract: Cabbage, is one of the most important crops of the cole group of vegetables. In India it ranks next to cauliflower in acreage and first in production among cole crops occupying an area of 3,72,000 ha with annual production of 8534,000 tons. It covers about 4.3% area under vegetable crops in India. In West Bengal cabbage covers 78200.00 ha of area and the total production is 2197400.00 MT. Black rot is a major disease of cabbage (*Brassica oleracea* var. *capitata*), caused by *Xanthomonas campestris* pv. *campestris* (Xcc). The disease has been observed in all cabbage growing areas of Bolpur, Birbhum, West Bengal. The present study was carried out on the pathology of black rot disease of cabbage. Morphological, cultural, biochemical, and physiological characteristics of the pathogen were studied. The bacterium produced small, yellow, circular, entire, smooth and shining colonies in the culture medium. The optimum temperature for the growth was found 30°C and white light supported maximum growth of the bacterium. Nutritional studies revealed that sucrose gave maximum growth followed by maltose, lactose, dextrose and fructose as the carbon source in the nutrient broth. Black rot of cabbage pathogen also infected other crops of crucifereae family such as Cauliflower, Knol khol, Mustard, Radish and Rape seed. These findings regarding the pathogen may help to formulate the more appropriate way and judicious application of different management options against the disease in this zone.

Keywords: Black rot, Cabbage, Pathology, *Xanthomonas campestris* pv. *campestris*

INTRODUCTION

Cabbage, (*Brassica oleracea* var. *capitata* L) a member of family *Brassicaceae*, is one of the most important cole crops. It is a rich source of vitamin A, B, C and minerals like phosphorus, potassium, sodium and iron. In India it ranks next to cauliflower in acreage and first in terms of production among cole crops occupying an area of 3,72,000 ha with annual production of 8534,000 tons (Anonymous, 2013). In West Bengal cabbage covers 78200.00 ha and the total production was 2197400.00 MT (Anonymous, 2014). The plant pathogenic bacterium *Xanthomonas campestris* pv. *campestris* (Xcc) causes black rot in a large number of crucifers worldwide (Alvarez, 2000). Advanced systemic infections can cause darkened leaf veins and stem vascular tissue, extensive leaf yellowing, leaf wilting and necrosis (Popovic' *et al.*, 2013). *X. campestris* pv. *campestris* is the most important and widely distributed plant pathogenic bacterium on brassicas. It is the causal agent of black rot, a vascular bacterial disease, and commercially one of the most important diseases on brassicas (Janse, 2005.) Mahiar and Khalif (1999) reported that *X. campestris* pv. *campestris* attacks all members of cruciferaceae family. The black rot disease in India was first reported on

cabbage from Bombay, Maharashtra (Patwardhan, 1928). Presently it is found in all parts of the country including West Bengal, Maharashtra, UP, HP, Rajasthan, Delhi and Meghalaya (Singh and Dhar 2010). Black rot caused by *X. campestris* pv. *campestris* is the major constraint to cabbage production in tropical areas. Diseased crops have a poor market value, and are unsuitable for storage as they quickly rot after harvest and yield losses of up to 100% have been experienced (Walangululu and Mushagalusa, 2000; Massomo *et al.*, 2003). Akhtar (1989) identified the pathogen causing black rot on cabbage as *X. campestris* pv. *campestris* based on the biochemical, physiological and pathogenicity characteristics of the isolate from cabbage. This is thought to be the first report of this pathogen on cabbage from Islamabad, Pakistan. The aim of the present work was to investigate the cultural, morphological, physiological and biochemical characteristics of the causal bacterium in the said zone.

MATERIALS AND METHODS

Isolation and purification of the pathogen

Selection of plant materials: For isolation of bacterial plant pathogens, at first the selection of the material was done appropriately. From young developing lesions the bacteria was isolated. Wherever, the

diseased specimen did not contain young lesions, the young lesions were produced by artificial inoculation on to the healthy host plant and those lesions were used for isolation. Cabbage leaves showing symptoms of bacterial blight were collected from the Agricultural Farm of Palli Siksha Bhavana (College of Agriculture), Sriniketan, Visva-Bharati, Birbhum, West Bengal. The presence of bacterium was confirmed by performing the ooze test under the microscope.

Preparation of suspension for isolation: To isolate the bacterium, diseased portion adjacent to the healthy tissue were cut, surface sterilized for 1 min. in freshly prepared 0.1% HgCl_2 solution and washed thoroughly thrice with sterile distilled water. The leaf bits were then transferred separately into a few drops of sterile water on a sterilized glass slide in an aseptic condition. The diseased bits were given a cut with sharp sterilized blade. The cut bits were left for few minutes to allow bacterial ooze to come out in the water. The suspension thus prepared was used for isolation.

Streaking the plates and slants: About 20 ml of the molten nutrient agar was poured in each Petri plates and was allowed to solidify for 1 hrs. After that the plates were kept upside down. After 2-3 hrs these plates were used for isolation purpose. A loopful of the suspension prepared as in the preceding section, was streaked over the agar surface by the inoculation needle in a zig zag fashion. Two more plates were streaked without recharging the needle with bacterial suspension. Similarly culture slants were also prepared. The plates and the slants were labeled and were incubated at $28 \pm 2^\circ \text{C}$ in a B.O.D. incubator and were examined daily.

Inoculums preparation: To maintain uniformity of inoculums, the bacteria growing on yeast extract glucose chalk agar slants, were gently brought into suspension by adding 10 ml of sterile distilled water per culture tube and scrubbing the bacterial growth. The suspension so obtained was centrifuged at 5000 rpm for 10 min, supernatant discarded and the pallette was re-suspended and the process was repeated thrice by adding fresh 10 ml of sterile distilled water after each centrifugation. The finally washed suspension was used as inoculums.

Characterization and identification of bacterium: For understanding and identification of bacterium, its morphological, cultural, physiological and biochemical characteristics were studied following standard procedure (Pelezar, *et al.*, 1957; Dowson, 1957; Dye, 1962; and Ryu, 1980).

Morphological characteristics: Following staining methods were used to study the morphological characteristics of bacterial cells.

Gram's differential staining: A thin bacterial smear was prepared from one day old culture on grease free glass slide. It was air dried, heat fixed and covered with ammonium oxalate crystal violet (primary stain) for one min. and washed in tap water for not more than

2 sec. Gram's iodine solution was then applied for one min. and washed in tap water. Ethyl alcohol was added drop by drop to wash the primary stain and counter stained with Safranin for 30 sec. Then washed in tap water, air dried and examined under oil immersion objective lens of a compound microscope (Dowson, 1957).

Negative staining of bacteria: In cytological studies it is very important to obtain more accurate picture of bacterial cell. This can be achieved by negative staining or relief staining. A suspension of a 24 h old bacterial culture was prepared in sterile water. A small drop of nigrosin solution (10% w/v) close to the end of a grease free slide was placed and a loop full of the bacterium was poured into the drop of stain. Both were mixed thoroughly with the help of a thin glass rod. A slide was placed against the drop of suspended bacteria at an angle of 45° and the drop was allowed to spread along the edge of the applied slide. The slide was air dried and was examined under oil emersion objective of a light microscope.

Cultural characteristics: The bacterium was grown on nutrient agar, potato dextrose agar and yeast extract glucose chalk agar in Petri plates. Inoculated Petri plates were incubated at $28 \pm 2^\circ \text{C}$. After 3 days of incubation, characters of bacterial colonies were studied under stereoscopic microscope. The colonies were observed up to 10 days.

Biochemical characteristics: The biochemical characteristics of bacterium causing black rot of cabbage, were determined by following different tests. Fresh (48 hrs old) bacterial culture was used for all the tests.

Starch hydrolysis: Inoculation was made at three places on starch agar medium in Petri plates. Incubated at $28 \pm 2^\circ \text{C}$ for 7 days and then flooded with Lugol's iodine solution (Iodine 1 g, KI 2g, distilled water 300 ml). The formation of clear zone around the growth of bacterium indicated the hydrolysis of starch (Pelezar *et al.*, 1957).

Gelatin liquefaction: Inoculation were made at different spots on gelatin medium in Petri plates and were, incubated at $28 \pm 2^\circ \text{C}$ for 2 days. These Petri plates were flooded with 8-10 ml mercuric chloride solution (HgCl_2 15g, conc. HCL 20 ml distilled water 100 ml). Liquefaction of gelatin was indicated by clear zone around bacterial growth (Pelezar *et al.*, 1957).

Hydrogen sulphide production: Dry sterilized filter paper strips soaked in saturated lead acetate solution, were suspended in inoculated peptone water tubes along with cotton plugs and incubated at $28 \pm 2^\circ \text{C}$ for 14 days. The blackening of strips indicated liberation of H_2S .

Physiological characteristics: The effect of temperature and light on the growth of the bacterium was studied for the characterization of the bacterium under investigation.

Effect of temperature: To study the effect of different

Table 1. Cultural characteristics of *Xanthomonas campestris* pv. *campestris* on different solid media after three days of incubation at 28±2°C.

Colony characters	Nutrient agar	Potato dextrose agar	Yeast extract glucose chalk agar
Colour	Pale lemon yellow	Empire yellow	yellow
Form	Circular	Circular	Circular
Margin	Entire smooth	Entire smooth	Entire smooth
Elevation	Convex	Flat	Convex
Size	10 mm	7 mm	10 mm
Pigmentation	Light yellow	Light creamy yellow	yellow

Table 2. Biochemical Characteristics of the black rot of cabbage causing bacterium *Xanthomonas campestris* pv. *campestris*.

S.N.	Biochemical characteristics	Result
1.	Starch hydrolysis	Positive
2.	Gelatin liquefaction	Positive
3.	Hydrogen sulphide production	Positive

Table 3. Growth of *Xanthomonas campestris* pv. *campestris* after 60 h of incubation on basal medium at different temperatures.

Temp ^r (°C)	Optical density (absorbance)
20	0.0065
25	0.0262
30	0.0377
35	0.0262
SE(treatment mean)	= 0.000716
CD at 5%	= 0.002335
CV	= 5.430480

Table 4. Effect of different lights of colour on the growth of *Xanthomonas campestris* pv. *campestris* after 60 h of incubation at 28±2°C.

Light source	Optical density (absorbance)
White	0.033
Red	0.021
Yellow	0.032
Green	0.019
Blue	0.003
Complete dark	0.003
Alternate light and dark	0.027
SE(treatment mean)	= 0.001846
CD at 5%	= 0.005599
CV	= 15.751473

temperatures on the growth of bacterium, four different temperatures were taken ranging from 20°C to 35°C with an interval of 5°C. Twenty ml nutrient broth was dispensed in each 100 ml conical flasks, autoclaved and inoculated with 0.2 ml of bacterial suspension. One flask from each treatment was kept as blank. Bacterial growth in terms of optical density was measured at 620 nm. wave length of U.V. light after 60 hrs of incubation at different temperature .

Effect of light: To investigate the effect of different light on the growth of bacterium, transparent

cellophane paper of different colours viz., colourless, yellow, green, blue, red and carbon paper for complete darkness were used. 100 ml conical flasks containing 20 ml of the nutrient broth were autoclaved, inoculated and wrapped with particular coloured paper and kept under artificial light source (100 watt bulb) along with control without inoculation. The bacterial growth in term of optical density (absorbance) was measured after 60 h of incubation at 28±2°C .

Nutritional Characteristics

Carbon sources: To study carbon requirement, the different carbon sources (dextrose, sucrose, maltose, fructose and lactose) were used to prepare the nutrient broth medium. The pH of the medium was adjusted to 7.0 for each treatment. 100 ml conical flasks containing 20 ml of the nutrient broth prepared with different carbon sources were autoclaved. One ml of bacterial suspension was then added to each treatments in an aseptic condition and the resultant growth was measured in terms of optical density (absorbance) after 72 hrs of incubation at 28±2°C .

Host range study: To determine the host range of *X. campestris* pv. *campestris*, 10 different plant species, belonging to three different families, were inoculated through carborundum abrasion technique and the disease development was recorded, if any. Two months old leaves were selected for inoculation suitable controls were maintained in each case.

RESULTS AND DISCUSSION

Morphological characteristics: The morphology of bacterial cell was studied with different staining techniques i.e. Gram staining and negative staining of bacteria. The methods used are described in detail under materials and methods. The bacterium produced straw yellow, smooth, glistening colonies with entire margin and convex elevation on NA medium. The study clearly indicated that *X. campestris* pv. *campestris*, black rot of cabbage pathogen, was gram-negative, short rod, mostly single and rarely in chains. Patel *et al.* (1951) and Orellana (1965) reported that the cells of *X. campestris* were rod shaped with rounded ends, borne singly or in short chains, 0.8x1.7µm in size, gram negative, capsulated, uniflagellate, and non spore forming. Gram-negative, Cells are straight rods, usually within the range 0.4-0.7 wide x 0.7-1.8 µm long, predominantly single. which are

Table 5. Different carbon sources on the growth of *Xanthomonas campestris* pv *campestris* after 72h of incubation at 28±2°C.

Carbon sources	Optical density(absorbance)
Dextrose	0.076
Sucrose	0.118
Maltose	0.096
Fructose	0.072
Lactose	0.081
SE(treatment mean) =	0.004835
CD at 5% =	0.015235
CV =	9.459222

same as present findings. Balan *et al.* (2014) The bacteria *X. axonopodis* pv *dieffenbachiae* produced slimy yellow colonies and were aerobic gram negative rods. It produced H₂S, liquefied gelatin and hydrolyzed starch. *X. campestris* pv. *campestris* (Pam.) Dowson. It is a small, rod shaped, aerobic, gram negative, non-spore forming bacterium described by Gupta *et al.* (2014).

Bacterium was rod shaped, Gram negative, single polar flagellate, capsulated and non spore forming. All the morphological characteristics of the bacterial isolates are similar to the genus *Xanthomonas* (Dye 1968).

Cultural characteristics: After 48 h of incubation at 28±2°C bacterial growth appeared on all three different media as minute and transparent colonies. The colonies became light yellow on nutrient agar and yeast extract glucose chalk agar and light creamy yellow on potato dextrose agar media after 72 h of incubation. The size and colour of bacterial colonies on different solid media after three days of incubation at 28±2°C are presented in Table 1. The similar results were obtained by Hayward (1983), Mariano and Gama (2005) and Viana (2006) on nutrient agar medium. The *Xanthomonas* colonies were yellow and gummy on nutrient agar but colourless on potato dextrose agar media (Orellana, 1965).

Biochemical characteristics: Biochemical characteristics of the black rot of cabbage pathogen were

studied as mentioned in materials and method and observations on various tests were given in Table 2. The results showed that the black rot causing pathogen *X. campestris* pv. *campestris* hydrolyzed starch and could liquefy the gelatin. The pathogenic bacterium also produced hydrogen sulphide in peptone medium as confirmed by the blackening of the paper strip. Which was similar as described by (Radunović and Balaž, 2012). The present result was also supported by the results found earlier by Jambenal *et al.* (2011). The bacterium had a single polar flagellum and it was catalase positive, hydrogen sulphide positive, oxidase negative and did not produce nitrate or indole as reported by Gupta *et al.* (2014). Naqvi *et al.* (2013) reported that the isolates of *X. campestris* pv. *campestris* tested for H₂S production giving a black discoloration on lead acetate paper strips. This was similar to present study.

Physiological characteristics: Physiological characteristics of the black rot of cabbage pathogen were studied as mentioned in materials and method and observations are follows:

Effect of different temperatures: Temperature has a great influence on the rate of growth of living organisms including the bacteria owing to its effect on the chemical and physical processes involved in growth. To find out optimum temperatures for the growth of the bacterium, four different temperatures ranging from 20°C to 35°C with an interval of 5°C were set. Bacterial growth in terms of optical density was measured after 60 h of incubation at different temperatures. The average results obtained were presented in Table 3. Thimmegowda (2006) observed that isolates of *X. oryzae* pv. *oryzae* tested at different temperature levels showed maximum growth at 30°C to 35°C and the temperature of 10°C and 40°C supported the poor growth. The data in Table 3 indicated that the bacterium *X. campestris* pv. *campestris* could grow over a wide range of temperature from 20°C to 35°C. The growth of bacterium gradually increased up to 30°C beyond which it decreased. The optimum tempera-

Table 6. Host range of *Xanthomonas campestris* pv *campestris* determined by inoculating two month old plants of different species using carborundum abrasion method.

Name of Host	Reaction	Incubation period (days)
Crucifereae		
Cabbage (<i>Brassica oleracea</i> var. <i>capitata</i>)	+	5-6
Cauliflower (<i>Brassica oleracea</i> var. <i>botrytis</i>)	+	5-6
Knol khol (<i>Brassica oleracea</i> var. <i>caultrapa</i>)	+	6-7
Mustard (<i>Brassica nigra</i>)	+	6-8
Radish (<i>Raphanus sativus</i>)	+	6-8
Rape seed (<i>Brassica napus</i>)	+	7-8
Solanaceae		
Chilli (<i>Capsicum annum</i>)	-	-
Tomato (<i>Lycopersicon esculentum</i> L.)	-	-
Leguminoceae		
Pea (<i>Pisum sativum</i> var. <i>arvense</i>)	-	-
Arhar (<i>Cajanus cajan</i>)	-	-

ture for growth was found 30°C. It was also reported that the organism was found to grow well in between 27-35°C with optima in between 28-30 °C which supported the present observation. Similar study was conducted by Dye and Lelliott (1974), on the pathogen *Xanthomonas oryzae* pv. *oryzae* required 25°C to 27°C as optimum temperature for the good growth and could grow up to 30°C.

Effect of light: To study the effect of different colour of light on the growth of the bacterium, transparent papers of different colours viz. white, red, yellow, green, blue and carbon paper for complete darkness were used. The optical density was measured after 60 h of incubation at 28±2°C and average results are presented in Table 4. Among the five different lights, continuous yellow and white lights were better than red, green and blue lights for the growth of the bacterium. The growth was minimum under complete darkness and very less under blue light. Bacterial growth was moderate when incubated under alternate light and dark.

Nutritional requirement

Effect of different carbon sources on the growth of the bacterium: Carbohydrates are important for microorganisms as they supply energy and various basic units of molecules required for growth. However a great diversity has been observed in microorganisms. During present investigation five different carbon sources, were used individually to prepare the nutrient broth medium with equivalent amount of carbon as specified in materials and method. The resultant growth after 72 hr in terms of optical density was recorded and presented in Table 5. The present data showed that the growth of the bacterium was highest in the medium with sucrose as the carbon source (0.118). The moderate growth of the bacterium was found in media with maltose (0.096) and lactose (0.081) as carbon sources. Tanaka (1964) reported that sucrose and glucose were the best carbon sources followed by fructose, galactose, mannitol and mannose. Xylose, lactose and starch were the poor carbon sources which were similar to the present investigation.

Host range: The pathogenic ability of *X. campestris* pv. *campestris* was tested on 10 plants species, belonging to three different families. Two month old leaves of test plant species were inoculated with bacterial suspension by carborundum abrasion technique. The inoculated plants were observed daily up to 30 days and disease development were recorded. The bacterium causing black rot of cabbage could produce visible symptoms only in plant species belong to the family cruciferae. The symptom expression took longer period of incubation (6-8 days) in Knol khol, Mustard, Radish and Rape seed as compared to cabbage and cauliflower Table 6. The bacterium attacked all members of cruciferae family including rutabaga, cabbage, savoy cabbage, broccoli, turnip, radish, kohlrabi, *Lapidium sativum*, *Raphanus sativus* var. *caudatus*, *Brassica juncea*, fodder cabbage, *Capsella bursa-pastoris* and *Cantella asiatica* (Mahiar

and Khalif, 1999)

Conclusion

Bacterial growth appeared, on all three different media, as minute and transparent colonies. The colonies became light yellow on nutrient agar and yeast extract glucose chalk agar and light creamish yellow on potato dextrose agar media after 72 h of incubation. Continuous yellow and white lights were better than red, green and blue lights. The growth was minimum under complete darkness and very less under blue light. Bacterial growth was moderate when incubated under alternate light and dark. The growth of the bacterium was highest in the medium with sucrose as the carbon source. The moderate growth of the bacterium was found in media with maltose (0.096) and lactose (0.081) as carbon sources. The cultural, physiological and biochemical characteristics of the bacterium under investigation found similar to that of *X. campestris* pv. *campestris*. These findings regarding the pathogen may help to formulate the more appropriate way and judicious application of different management options against the disease.

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