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Research Paper

Isolation and Identification of *Xanthomonas Campestris Pv. Campestris* Causing Black Rot on Cauliflower From Plains of Kerala

Agriculture

KEYWORDS : Black rot, Cauliflower, Xcc, Isolation

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ABSTRACT

Laboratory assay was conducted to detect and identify Xanthomonas campestris pv. campestris naturally infected cauliflower leaves and curds. The semi-selective me-dia, Potato Sucrose Peptone Agar (PSPA) medium was used to isolate the pathogen. Colonies of the pathogen were yellow, cir-cular, slimy, fluidal and convex colonies with entire margin on PSPA medium. Morphological, physiological and bio-chemical characters coupled with pathoge-nicity the pathogen was identified as Xan-thomonas campestris pv. campestris.

Introduction:

Cauliflower is one of the most popular vegetable among Cole crops. Though cauliflower is a cool season crop, with the introduction of tropical cultivars it can be cultivated even in plains. One of the most destructive diseases of cauliflower is black rot caused by *Xanthomonas campestris* pv *campestris.* This pathogen was isolated from the leaves and curds showing typical symptoms of black rot from plains of Kerala. The pathogen affects all the above ground parts throughout the growth stage. Initially chlorotic lesions which later changes to V shaped lesions, blackening of the veins, drying of the lesion, withering of the leaves were common symptoms seen on the plants infected locally. Dwarfing of the infected plant was not uncommon. Discoloration of vascular bundle also noticed. Systemically infected plants produce small curds. Infected curds show a slimy discoloration at the center and later infected by secondary soft rotting organisms (Mirik *et al*., 2008). No research work has been taken up on this pathogen which can cause disease even in plains where cauliflower cultivation was newly practiced.

Isolation of pathogen

The pathogen causing black rot of cauliflower was isolated from naturally infected leaves and curds collected from the field, showing typical symptoms (Mirik *et al*., 2008). The infected samples were washed thoroughly and the infected areas were cut into small bits, surface sterilized with 70% ethyl alcohol for a minute. These bits were then washed with three changes of sterile water and crushed on sterilized glass slide to get bacterial suspension. This suspension was streaked on Potato Sucrose Peptone Agar (PSPA) medium (Potato sucrose peptone agar medium; potato 300g, peptone, 2g; sucrose, 20g; KH2PO4, 0.2g; NaHPO4, 0.5g; calcium nitrate, 0.5g; KCl, 0.05g; ferrous sulphate, 0.05g; agar 20g per litre) to get single isolated colonies of the bacterium. Washed bits were also directly placed on PSPA medium. The plates were incubated for 48 h at room temperature. Characteristic single colonies were selected on the basis of its colour, fluidity and sliminess and purified by repeated streaking on PSPA medium so as to get single cell colonies. Then pure culture is maintained in slants as well as in sterile water under refrigerated condition.

Test for pathogenicity

A thick suspension of 24 h old culture of the isolate was inoculated on a susceptible cauliflower variety grown under controlled aseptic conditions in green house (25 days after transplanting). The plants were sprayed with water two hours before inoculation to provide favorable conditions for infection.

Inoculation of plants was made with a sterile needle giving pin pricks along the leaf margins. The culture was smeared on the pin pricked area by means of cotton dipped in bacterial suspension. The inoculated plants were covered with polythene cover. Five plants were inoculated for this study. The positive (inoculated plants) and negative (sterile water) controls were prepared in a similar manner.

Symptoms were recorded after 3-4, 10-13, 20 days and compared with positive and negative controls. Pathogenicity was evaluated on the occurrence of typical symptoms on leaves.

Identification of pathogen

The cultural, morphological, physiological and biochemical characters of the pathogen were studied following the methods using 24-48 h old cultures.

Culture characters:

The colony morphology was studied from 48 h old culture of the bacterium grown on PSPA medium. Colonies were observed for their colour, shape, size, elevation, margin and fluidity.

Pigment production: Production of water soluble and insoluble pigments by the isolates was studied by streaking on Yeast Glucose Agar and King's B medium.

Gram's reaction: For Gram's staining, 48 h old culture was used. Shape and grams reaction of bacteria was observed under oil immersion objective. KOH test was conducted to confirm the Gram's reaction.

Solubility in 3% KOH: A loopful of bacterial culture was placed on a clear glass slide. One drop of three per cent KOH solution was added, thoroughly mixed with the help of inoculation needle and moved up and down to know the solubility in KOH.

Endospore staining: A loopful of bacterial culture was taken and smeared on glass slide and fixed by heating, and then a few drops of 1.5 per cent amidoblack was added and allowed to stay for 70 sec. Then the slide was washed under gentle stream of running water, stained for 20 sec with 1 per cent carbol fuschin and washed thoroughly under tap water. Then the slide was blot dried and observed under microscope for endospore.

Mode of utilization of glucose: To determine whether the bacterium utilized glucose only under aerobic condition or both under aerobic and anaerobic condition, one per cent glucose was added to the prepared basal medium and dispensed in tubes upto 4 cm. The medium was sterilized by tyndalization and inoculated in duplicate by stabbing with straight inoculation needle charged with bacterial growth. In one of the tubes the medium was sealed with 1 cm layer of sterilized liquid paraffin. The tubes were incubated at room temperature and observations on change in colour were taken at regular intervals up to 15 days.

Citrate utilization test: One day culture was streaked on the surface of Simmon's Citrate Agar and observed for any colour change in the medium.

Starch hydrolysis: The ability of the bacterium to hydrolyze starch was tested using Nutrient Agar Medium containing 0.2 per cent soluble starch. Test organism was spotted on Petri plates containing medium. The dishes were flooded with Lu-

gol's iodine solution after 48 h of incubation and observed for the colour change.

Catalase test: Smear of one day old culture grown in PSPA medium was prepared on clear glass slide and covered with few drops of three per cent H_2O_2 and observed for the formation of effervescence.

Denitrification test: Bacterial culture was stab inoculated into the Vanden Mooter Succinate Medium and sealed with three ml of one per cent molten agar and examined daily for production of gas under the seal.

Oxidase test: The 24 h old bacterial culture was spot inoculated on oxidase disc and the change in colour of the disc from white to purple or blue within 60 sec was observed.

Arginine dihydrolase reaction: The bacterial culture was stab inoculated into the semisolid medium of Thornley and the tubes were incubated at room temperature for seven days and observed for colour change.

Production of hydrogen sulphide: The ability of bacterium to liberate hydrogen sulphide was tested using peptone water medium. Five ml of medium was dispensed in test tubes and autoclaved. Lead acetate paper stripes of 5 x 50 mm size were prepared by soaking them in super saturated solution of lead acetate. The stripes were dried, autoclaved and again dried. The tubes were inoculated in triplicates with bacterial isolates and lead acetate stripes were inserted aseptically by the side of the plug in the tube. The tubes were incubated at room temperature and observations were recorded at regular intervals up to 14 days for blackening of test strip.

Methyl Red Test: Five ml of methyl red broth medium was dispensed in tubes and sterilized by steaming for 30 seconds for three successive days. Tubes were then inoculated with 48 h old culture of bacterial isolate. The tubes were incubated for seven days at room temperature. Few drops of 0.02 per cent methyl red in 50 per cent alcohol was added to culture tube and observed for colour change.

Gelatin liquefaction: Sterilized nutrient gelatin medium was spot inoculated with 48 h old culture of bacterium. After incubation of seven days agar surface was flooded with 0.2 per cent $HgCl_2$ solution in dilute HCl and observed for the clear zone around the bacterial growth.

Production of Indole: Tryptophan broth medium was used for this test. The medium was dispersed in tubes and autoclaved. Oxalic acid test stripes were used for detecting indole production. Filter paper stripes of size 5 x 50 mm were soaked in warm saturated solution of oxalic acid and cooled. When the stripes got covered with oxalic acid, they were dried at room temperature and used without sterilization. The tubes were incubated with bacterial isolate and oxalic acid stripes were inserted into the tube by the side of the plug, incubated and observed regularly for 14 days. Observed for change in colour of oxalic acid crystals on test strip to pink or red which indicates indole production.

Growth on 6 % NaCl: Peptone water with six per cent NaCl was used for the test. The medium was dispensed in tubes; autoclaved and inoculated with bacterium and incubated. The ability of bacterium to grow on medium was observed.

Lipolytic activity: Sierra's medium was employed for this test. The medium was dispersed in 99 ml quantities in flasks, autoclaved and cooled to 45 ^{0}C. One ml of Tween 80 was added to the medium and thoroughly mixed. The medium was poured in sterile petridishes and the test bacterium was spot inoculated on the medium. The plates were incubated and observed at regular intervals for 15 days. Observed for opaque zone around the bacterial growth which indicates positive lipase production.

Utilization of Carbon sources: Basal medium for xanthomonads was supplemented with one per cent concentrated solution of carbon compound *viz,* dextrose, fructose, sucrose, glucose, maltose, lactose, mannose, cellobiose, arabinose, adinitol, inositol, glycerol, mannitol, dulcitol and sorbitol. 0.7 ml of five per cent alcoholic solution of bromocresol purple was added to get reddish violet colour. Medium was sterilized by tyndalisation and slants were inoculated with bacterium, incubated at room temperature. Observed for the change in colour of medium from reddish violet to yellow, which indicates the production of acid from carbon compounds.

Production of Ammonia: Peptone water was used for the test. The culture was inoculated in peptone water and incubated for 48 h. The accumulation of ammonia is detected by Nessler's reagent which gives brown to yellow precipitate with ammonia.

Results:

The bacterium gave rise to yellow, circular, slimy, fluidal and convex colonies with entire margin on PSPA medium. A non water soluble yellow pigment on yeast glucose chalk agar medium was produced by the bacterium. The bacteria were found to be gram negative, short rods and produced no endospores. The bacterium was found to utilize glucose oxidatively (aerobically) since the medium in the open tubes turns yellow from the top. It utilized citrate as a source of carbon which was evidenced by the change in colour of the medium. Hydrolyzed starch as indicated by colorless zone around the bacterial growth in contrast to the outer blue back ground of the medium. Positive to catalase reaction, arginine hydrolase as indicated by the change in colour of the medium to red liberated H_2S within 14 days which is evidenced by blackening of the lead acetate strip. Liquefied gelatin in the plates which were inoculated with the bacterium within a week. Produced lipases - observed by formation of opaque zone around the bacterial growth. The bacterium produced ammonia which is evidenced by the accumulation of yellow precipitate when Nessler's reagent was added to the peptone water inoculated with the bacterium after 48 h. Negative to methyl red test as evidenced by the absence of the development of distinct red colour in the culture tube when few drops of 0.002 per cent methyl red in 50 per cent alcohol was added. Nitrate was not produced as shown by the absence of gas production under the seal of molten agar in the test tube. The oxalic acid crystals on the strip did not turn pink or red which indicated that the bacterium did not produced indole. No growth of bacterium in 6 per cent NaCl was noticed. Of the 15 carbon compounds tested (Table 1), the bacterium produced acid from mannose, xylose, fructose, glucose, maltose, lactose, sucrose, dextrose and arabinose as indicated by the change of colour of the medium from reddish violet to yellow. There was no change in the colour of the medium from reddish violet to yellow in tubes containing sorbitol, inositol, cellobiose, adinitol, glycerol, dulcitol and mannitol.

Pathogenicity test:

The presence of pathogen was conformed by pathogenicity. The symptoms of the disease on leaves appeared on the third day of inoculation (25 day old transplanted seedlings) near the pin-pricked region at the point of entry.

Initially the symptom was seen as chlorotic flecks, developing into a chlorotic lesion, which enlarged in size and occupied larger area. The veins started to show discoloration. Within two to three days formation of 'V' shaped lesion was observed. Later, it turned brown and black and finally into a necrotic lesion. The death of the tissues resulted in brittleness of leaf. Reisolation from the infected leaves resembled the original isolate.

Discussion:

The bacterium causing black rot of cauliflower *Xanthomonas campestris* pv. *campestris* was isolated from diseased leaves and curds with typical symptoms. The colonies were yellow colored mucoid, slimy, glistening, convex and round colonies were obtained (Bradbury, 1984; Schaad and Stall, 1988; Guptha and Thinol, 2006 and Romero *et al*., 2008).

The yellow colonies obtained on isolation were identified as *Xanthomonas campestris* pv. *campestris* on the basis of colony

and morphological characters, staining reaction, physiological and biochemical characteristics as per the methods described by Bradbury, (1984); Harringan and Mc Cane (1996) and Schaad *et al.* (1988).The bacterial cells were small straight rods (0.4-0.7 x 0.7-1.8 µm long) gram negative, aerobic, motile by single polar flagellum, produces no endospore. Colonies are mucoid, convex and shiny on YDC and NGA. Non water soluble pigments were produced on YDCA medium. They were weak producers of acids from carbohydrates. The different physiological and biochemical characteristics such as utilization of glucose, maltose, fructose, dextrose, sucrose, mannose and arabinose and did not grow on media containing sorbitol, inositol, cellobiose, adinitol, glycerol, dulcitol and mannitol. The isolate hydrolyzed starch, liquefied gelatin, produced hydrogen sulphide, positive to catalase reaction, did not produce indole and negative to urease and methyl red reaction, did not reduce nitrate to nitrite, growth at 6 per cent NaCl was absent. Thus, based on cultural, morphological and biochemical characters coupled with pathogenicity, the bacterium causing black rot of cauliflower is tentatively identified as *Xanthomonas campestris* pv. *campestris*.

In this study isolation of pathogen was made from naturally infected cauliflower leaves and curds grown in plains of Kerala. The experiment confirmed the presence of *Xanthomonas campestris* pv. *campestris* on cauliflower cultivated even in plains .

Table: 1 Cultural, morphological and biochemical characters of the pathogen

Sl. No.	Cultural, morphological & biochemical characters	Observation	Sl. No.	Cultural, morphological & biochemical characters	Observation
1	Grams reaction	-ve	17	Utilization of carbon compounds with acid production	
2	Margin	Entire	a	Glucose	+ve
3	Surface	Small smooth	b	Maltose	+ve
4	Configuration	Rod	c	Lactose	+ve
5	Pigment production		d	Fructose	+ve
a	Water soluble	-ve	e	Dextrose	+ve
b	Non water soluble	+ve	f	Sucrose	+ve
6	Mode of utilization of glucose		g	Mannose	+ve
A	Aerobic	+ve	h	Arabinose	+ve
b	Anaerobic	-ve	i	Sorbitol	-ve
7	Citrate utilization test	+ve	j	Inositol	-ve
8	Starch utilization	+ve	k	Cellobiose	-ve
9	Production of H_2S	+ve	l	Adinitol	-ve
10	MR test	-ve	m	Glycerol	-ve
11	Gelatin liquification	+ve	n	Dulcitol	-ve
12	Production of indole	-ve	o	Mannitol	-ve
13	Nitrate reduction	-ve	18	Ammonia production	+ve
14	Catalase test	+ve	19	Urease test	-ve
15	Growth in 6 % NaCl	-ve	20	Arginine dihydroginase	-ve
16	Lipolytic activity	+ve			

+ve-Positive; -ve-Negative

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