Environment and Ecology 40 (4A) : 2296—2304, October—December 2022
ISSN 0970-0420

Biochemical Characterization of *Xanthomonas campestris* pv. *campestris* and ITS Eco-Friendly Management by Native Rhizobacteria

Rahul Patidar, R.K. Ranjan

Received 12 July 2022, Accepted 19 August 2022, Published on 4 November 2022

ABSTRACT

Black rot disease is a serious problem in the development of cauliflower, which is one of world's most popular winter vegetables. The pathogen of black rot disease was identified as *Xanthomonas campestris* pv. *campestris* through morphological and biochemical characteristics. Pathogen isolates developed yellow colored, convex-shaped and viscous or sticky colonies on NSA medium regards to morphological characters. The biochemical studied shows that *Xcc* is small rod shaped, gram negative, gave positive test for KOH string test and catalase test and negative test for indole test and nitrate reduction test. *Xcc* utilize lactose, maltose, fructose, dextrose, mannose, glycerol, rhamnose, cellobiose, melezitose, sucrose and L- arabinose as a carbon source. Evaluation of 30 rhizobacteria isolates against *Xcc* under *in vitro* shown that, 3 isolates were found effective and developed inhibition zone of more than 10 mm and RAC 3 isolates developed a maximum inhibition zone of 27.03 mm.

Keywords Black rot, Biochemical test, *Brassica oleracea* var. *botrytis*, Rhizobacteria.

INTRODUCTION

Cauliflower (*Brassica oleracea* var. *botrytis*) is one of the important winter vegetable crops belongs to the family Brassicaceae and cultivated throughout the world. Cauliflower occupies an important place among all the vegetables in India as well as in world. Cauliflower is a good source of vitamin-C, vitamin-B6, dietary fiber, glucosinolates, carbohydrates, proteins and trace amounts of other vitamins and minerals which improves digestive health, reduce inflammatory issues, oxidise fats, improve immune system and also reduces the development of cancer like diseases (Sarikamis 2009).

Infectious diseases like black rot, club root, seedling damping-off, fungal wilt and downy mildew reduces the quality and productivity of cauliflower. Among all these biotic problems, black rot is major problem throughout world. The causal pathogen of black rot disease of cauliflower is *Xanthomonas campestris* pv. *campestris* (Pammel) Dowson (*Xcc*),

Rahul Patidar [1]*, R.K. Ranjan [2]
[2] Assistant Professor
Department of Plant Pathology, Dr Rajendra Prasad Central Agricultural University, Pusa, Samastipur, Bihar 848125, India

Email: patidarrahul047@gmail.com
*Corresponding author

which prevalent in tropical and subtropical regions of the world. Initial symptoms of the black rot disease include development of water soaked chlorotic lesions along the leaf edges which progresses towards the midrib becomes "V" shaped and later it becomes necrotic lesion (Vicente *et al*. 2001). On seedlings stage, chlorotic and necrotic lesions appear on the cotyledons or on the lower leaves, which becomes blacken and eventually die and fall off (Jensen *et al*. 2010).

The bacterium *X. campestris* pv. *campestris* (Pammel) Dowson, microbe belongs to Eubacteria domain, belongs to phylum Proteobacteria, Gamma proteobacteria class, order of Xanthomonadales, and family Xanthomonadaceae. Under microscope, *Xcc* bacteria look straight rods with a diameter of 0.4-1.0 x 1.5-4.0 µm, are aerobic, motile due to *Monotrichous flagella* and develop yellow, mucoid, convex colonies on nutrient agar (Schaad *et al*. 2001). Prasanna and Ravi (2014) isolated *Xcc* bacterial pathogen causing black rot of cauliflower from infected leaves and bacterial colonies look circular, convex, slimy, fluidal, and yellow in nature when cultured on PSPA (Potato Sucrose Peptone Agar) medium. *Xcc* utilize citrate, starch, and glucose as carbon sources, bacteria also gave positive test for catalase test, produce hydrogen sulfide and also produce ammonia but failed for methyl red test, nitrate reduction and indole production. Similarly, Elsisi (2017) isolated 6 bacterial isolates from cabbage leaves showing typical black rot symptoms on nutrient medium and tested these isolates for the different biochemical tests, all give positive string test and catalase test; negative test for indole production, fat hydrolysis and methyl red test.

Many plant-associated soil/root rhizobacteria have established competitive, exploitative, or neutral relationships with plants; the plant and the rhizobacteria comprise the holobiont (Lyu *et al*. 2020). Researchers have recently begun to look into the possibility of using beneficial rhizobacteria to both reduce plant pathogenic effects and promote plant growth (Zhang *et al*. 2013, Qiao *et al*. 2017). Fallahzadeh-Mamaghani *et al*. (2021) isolated 524 rhizobacterial strains and *Paenibacillus polymixa* strain N179 found to very effective against *Xcc* under *in vitro*. Similarly, Tistechok *et al*. (2019) isolated rhizobacteria isolates from the soil and test the antimicrobial action against different fungal and bacterial plant pathogens, among them many isolates found effective against *Xcc* pathogen.

The aims of the present study are to isolate the plant pathogen *Xanthomonas campestris* pv. *campestris* isolates from cauliflower plants and characterise them using biochemical tests and utilization of various sugars as a carbon source. The *Xcc* plant pathogen has been found to be a threat to a wide range of *Brassica* plants. As a result, we explored the possibility of using rhizobacteria as a biocontrol agent to supress the pathogen under *in vitro* condition.

MATERIALS AND METHODS

Collection of isolates of *Xanthomonas campestris* pv. *campestris* (*Xcc*)

Cauliflower leaves with typical "V" shaped yellowing with blackened veins symptoms of disease were collected from the Samastipur district of Bihar. Diseased sections of leaves were sliced in each way that each cut section have 50% disease and 50% healthy portion. The cut portion of the leaf was surface sterilized for 2-3 minutes with a 0.1% mercury chloride solution, and washed several times with sterile purified water and placed on aseptic blotting paper to remove excess moisture. Later with the help of forceps cut sections were placed on solidified NSA (Nutrient Sucrose Agar) petri plates aseptically and incubated them at 28 ±1°C for 48 hours.

Pathogenicity test:

The pathogenicity experiment was carried out under protected glass house conditions, for that healthy diseased free cauliflower seedlings (30 DAS) were transplanted and after 20 days of transplanting, inoculation of *Xcc* pathogen in healthy cauliflower plant was performed. Sterile water was used to make the bacterial suspension. The suspension's concentration was held at about 10^8 CFU/ml. The inoculation was done in a healthy leaf by pinning method. Near the edge of the leaf tiny holes were created by a sterilized pin and then gently bacterial suspension was applied. In control plant, sterile distilled water was applied

and at daily intervals, the plants were observed for symptoms development.

Morphological and biochemical properties of *Xcc* isolates

Biochemical experiments were conducted for the characterization *Xcc* isolates. Gram staining, KOH string test, catalase test, nitrate reduction test, indole production and utilization of different carbohydrate sugars as carbon source.

Gram staining

A drop of sterile purified water was applied to the cleaned slide and then 48-hour-old culture of bacteria was picked and mixed thoroughly on the slide with the sterilized water before being scattered over a larger region of the slide to make a smear with the help of inoculation loop. The bacterial specimen then heat-fixed by passing the slide slowly over the flame twice, then add 2-3 drops of crystal violet to the smear for 60 seconds, the slide was rinsed for a few seconds with running tap water. Smear then treated with 2–3 drops of Gram's iodine for 60 seconds and rinsing the slide under running tap water. After that 95% pure ethanol was applied on the slide to decolorize the smear and again rinse with running water. At last the specimen was counterstained for about 60 seconds with safranin and rinse with running water then air dried, and examined under microscope at 100X using oil immersion.

KOH string 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.

Catalase test

On a clean glass slide, a loop of the 48-hour-old culture of the test bacterium *Xcc* was placed, then 2-3 drops of 3% hydrogen peroxide were applied to the slide having bacterium culture and allowed to react for a few seconds for gas bubbles formation.

Nitrate reduction test

NA broth having KNO_3 as a nitrate source was pour into the test tubes in 10 ml amounts, plugged and then sterilized in autoclaved and later kept it for cooling at room temperature. The medium was then inoculated with the 48-hour-old *Xcc* culture and each tube was plugged with non-absorbent cotton, one test-tube in which no *Xcc* inoculated serves as a control and all the test tubes were incubated at 28°C ±1°C. After 3rd day of incubation test tube were taken out and in each test tube 3-4 drops of sulphanilic acid and then dimethyl-alpha-naphthylamine was added gently. The colour transition was then noticed and if the color of the medium varies to pink or red, it means the nitrate reduction test is positive.

Indole test

The indole test was performed by taking 10 ml of sterilized peptone water in test tubes and inoculated with *Xcc*. For detection of indole formation, Himedia kovac's reagent strip was inserted between the cotton plug and inner wall of the test tube above the inoculated peptone water to observe indole formation by the organism. The test tube which was not been inoculated with *Xcc* mark as control. For 24-72 hours, test tubes were incubated at 35-37°C. The pink color at the bottom of the strip was deemed as positive.

Utilization of sugar as a carbon source

To characterize the *Xcc* bacterial isolate, several carbon source utilization tests were done with the use of the KB009 HiCarbohydrate Kit (HiMedia Laboratories Pvt. Limited), which contains several media wells that have sugars and sugar alcohols as a sole carbon source and when inoculated microbe utilizes the sugar and produces the alcohol color of the well changes from red to yellow. The bacterial suspension was made by mixing a loop of 48-hour-old *Xcc* culture in 5 ml of sterile distilled water to a concentration of 10^8 CFU/ml. By using the surface

inoculation approach, each well in the kit was loaded with 50µl of the bacterial suspension and sterile distilled water was used in the control well. After that, it was incubated at 28 ±1°C.

Isolation and screening of rhizobacteria against *Xcc*

Thirty soil rhizobacteria were isolated from 3 soil samples collected from the rhizosphere of the cauliflower plant. Rhizobacteria were isolated on King's B agar media and rhizobacterial isolates were given a strain number and were correctly label for easy identification for further use. For antagonistic test, one loop of 48 hours old ***Xcc*** culture were evenly spread over the NSA medium petri plates and with the help of cork borer, three well of 5mm diameter were made in each petri plate. After that, the suspension of each rhizobacterial isolate was prepared in sterilized water in the test tube and then with the help of a micropipette, each well was loaded with rhizobacteria suspension. For control, well in a plate was poured with sterile distilled water. The plates were then incubated for 48 hours at 28 ±1°C. Rhizobacteria isolates efficacy was recorded on the basis of inhibition zone development against ***Xcc***.

RESULTS AND DISCUSSION

Isolation of *Xcc*

After incubation for 2 days, yellow colored, convex-shaped and viscous or sticky colonies were developed near the cut portion of leaves and also a slimy

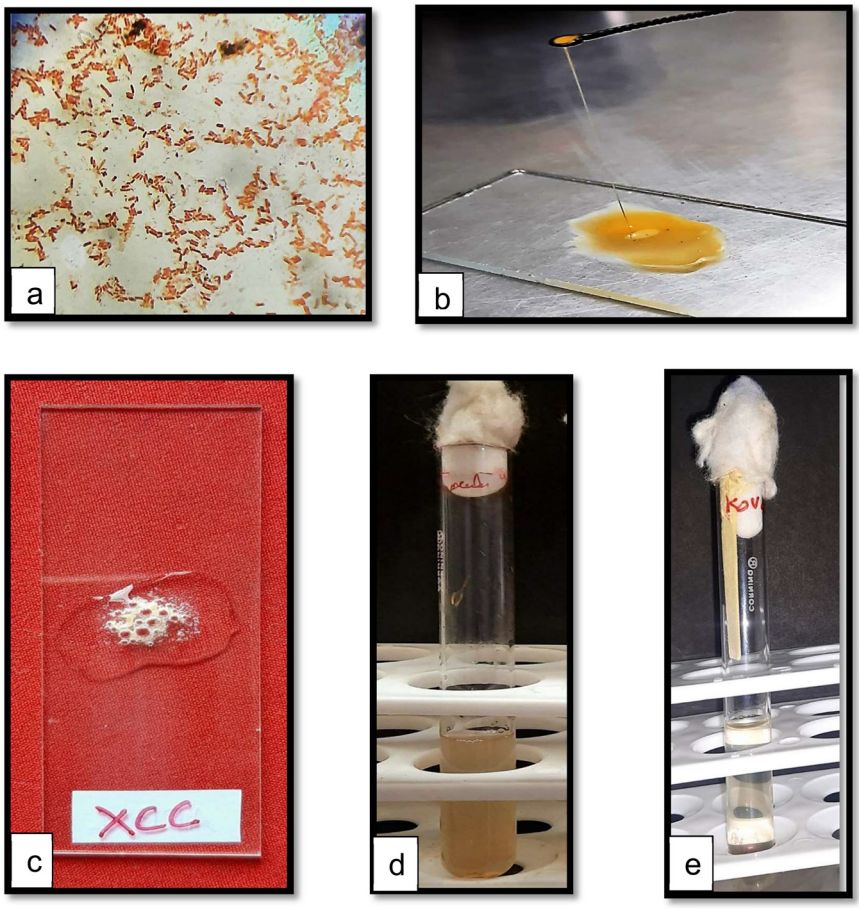

Fig. 1. a) Gram staining, b) KOH test, c) Catalase test, d) Nitrate reduction test and e) Indole test.

Table. 1. Result of biochemical test for *Xcc* isolate. '+' Positive test '-' Negative test.

Sl. No.	Biochemical test	Result
1.	Gram staining	-
2.	KOH string test	+
3.	Indole production	-
4.	Nitrates reduction	-
5.	Catalase test	+

mass of bacterial colonies was formed around the leaf portion and that single colony were streaked on new NSA petri plates. Similarly, Rathaur *et al*. (2016) isolated 47 strains of *Xcc* from different crucifer crop on NSA medium, all *Xcc* produced mucoid, raised, translucent with yellow colored colonies. Deivamani and Muthamilan (2016) isolated 10 strains of *Xcc* on NA (Nutrient Agar) and colonies were convex, circular and filiform.

Pathogenicity test

The development of disease symptoms starts from 16th days after inoculation, initially a small yellow lesion was developed at the edge of the leaf and gradually yellow lesion progresses upper side of the leaf forming a 'V' shaped lesion and leave a necrotic area behind it. Later on, blackening of the veins were developed and it is clearly visible. The symptoms were progress and spread all over the whole the leaf. A similar finding which supports the present study done by Prasanna and Ravi (2014). They inoculated pathogen by the pin-picked method in cauliflower plant and symptoms like chlorotic lesion and discoloration of veins developed after 3 days of inoculation. Elsisi (2017) find similar symptoms development on cabbage plants after 14 days of inoculation.

Morphological and biochemical properties of *Xcc* isolates

Gram staining

In gram staining, *Xcc* retains only light red colored stain (safranin) and that shows a negative test for gram staining. When studied microscopic pictures with the help of a microscope, bacteria visible as small rod-shaped and light red in color at 100 X magnification (Fig. 1a) (Table 1). The similar result was made by Deivamani and Muthamilan (2016), they found that *Xcc* is gram-negative and rod-shaped bacteria.

KOH string test

The bacterial colonies mixed with 2-3 drop of 3% KOH solution over a slide, bacterial colonies were looking soluble in solution and when a loop was dipped in that mixer and remove it above a small height, a thin thread or string was formed, which shows a positive test for KOH string test (Fig. 1b). Prasanna and Ravi (2014) reported that *Xcc* isolates formed string in KOH string test.

Table 2. The result of utilization of different sugars as a carbon source. '+' Positive for oxidation/utilization of sugar. '-' Negative for oxidation/utilization of sugar. '±' Slow oxidation/utilization of sugar.

Sl. No.	Test	Color of medium		+ or -
		Before incubation	After incubation (72h)	
1.	Lactose	Red	Yellow	+
2.	Xylose	Red	Red	-
3.	Maltose	Red	Yellow	+
4.	Fructose	Red	Yellow	+
5.	Dextrose	Red	Yellow	+
6.	Galactose	Red	Red	-
7.	Raffinose	Red	Red	-
8.	Trehalose	Red	Red	-
9.	Melibiose	Red	Red	-
10.	Sucrose	Red	Orange	±
11.	L-Arabinose	Red	Orange	±
12.	Mannose	Red	Yellow	+
13.	Inulin	Red	Red	-
14.	Sodium gluconate	Red	Red	-
15.	Glycerol	Red	Orange	±
16.	Salicin	Red	Red	-
17.	Dulcitol	Red	Red	-
18.	Inositol	Red	Red	-
19.	Sorbitol	Red	Red	-
20.	Mannitol	Red	Red	-
21.	Adonitol	Red	Red	-
22.	Arabitol	Red	Red	-
23.	Erythritol	Red	Red	-
24.	α Methyl-D-glucoside	Red	Red	-
25.	Rhamnose	Red	Yellow	+
26.	Cellobiose	Red	Yellow	+
27.	Melezitose	Red	Yellow	+
28.	α Methyl-D-mannoside	Red	Red	-
29.	Xylitol	Red	Red	-
30.	D-Arabinose	Red	Red	-
31.	Sorbose	Red	Red	-
32.	Control	Red	Red	-

Catalase test

The production of air bubbles within one minute was recorded after few drops of 3% hydrogen peroxide were applied on the *Xcc* culture which gives a positive reaction for the catalase test (Fig. 1c). Result depicted that *Xcc* is aerobic bacteria. Similarly, Maji and Nath (2015) reported *Xcc* isolates gave positive test for catalase.

Nitrate reduction test

The reduction of nitrate by bacteria was evaluated by culturing them in nitrate broth for 3 days and reduction of nitrate to nitrite was tested by adding 2-3 drops of sulphanilic acid and dimethyl-alpha-naphthylamine in 3 days old broth culture, development of red color shows the reduction of nitrate. But no color change was visible which give a negative reaction for the nitrate reductase test (Fig. 1d). The similar finding was made Naqvi *et al*. (2013) reported that *Xcc* isolates gave negative test for nitrate reductase activity.

Indole test

In this test, no color changes in the Kovac's reagent strip was visible which indicates that bacteria didn't produce indole and shows a negative test for indole production (Fig. 1e). Prasanna and Ravi (2014) reported that *Xcc* isolates were gave negative test for indole production. Results of morphological and biochemical test listed in Table 1.

Utilization of sugar as a carbon source

Color changing of the well was recorded in Table 2 and match with the chart provided with the Kit which indicates that *Xcc* isolates utilize lactose, maltose, fructose, dextrose, mannose, glycerol, rhamnose, cellobiose, melezitose and slow utilization of sucrose and L- arabinose as a carbon source (Fig. 2). A similar finding related to carbon utilization was done by Elsisi

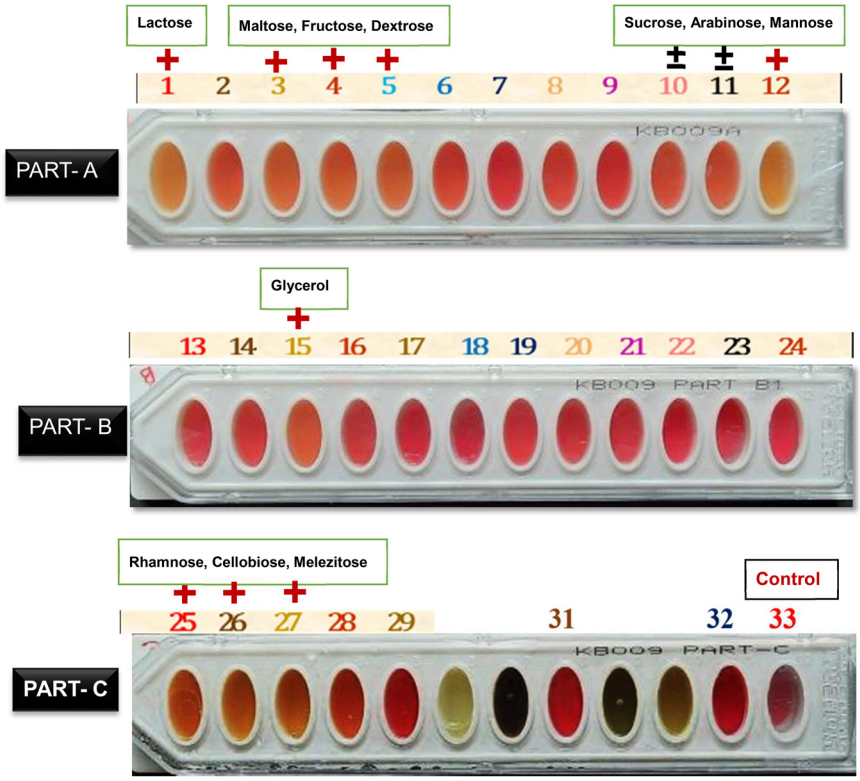

Fig. 2. Result of utilization of different sugars as a carbon source using KB009 HiCarbohydrate Kit.

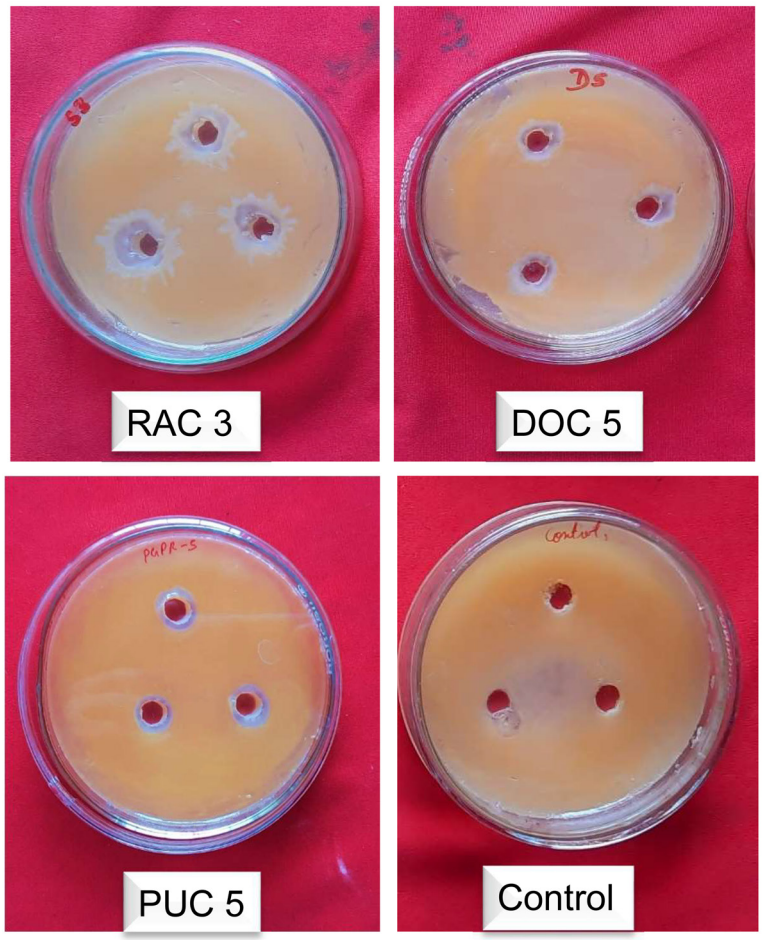

Fig. 3. Inhibition zone produced by different isolates of rhizobacteria against *Xcc*.

(2017), their study reveals that *Xcc* utilized lactose, dextrose and mannose as carbon sources, Popovic *et al*. (2014) also found that *Xcc* hydrolyzed esculin and utilized darabinose, maltose, mannose and sucrose as

Table 3. Inhibition zone developed by rhizobacteria isolates against *Xcc*.

Sl. No.	Rhizobacteria isolates	Inhibition zone (mm) of *Xcc* by rhizobacteria
1.	PUC 1	0
2.	PUC 2	0
3.	PUC 3	0
4.	PUC 4	0
5.	PUC 5	12.27
6.	PUC 6	0
7.	PUC 7	0
8.	PUC 8	0
9.	PUC 9	0
10.	PUC 10	0

Table 3. Continued.

Sl. No.	Rhizobacteria isolates	Inhibition zone (mm) of *Xcc* by rhizobacteria
11.	AIC 1	0
12.	AIC 2	0
13.	AIC 3	0
14.	AIC 4	0
15.	AIC 5	0
16.	DHC 1	0
17.	DHC 2	0
18.	DHC 3	0
19.	DHC 4	0
20.	DHC 5	19.03
21.	DHC 6	0

Table 3. Continued.

Sl. No.	Rhizobacteria isolates	Inhibition zone (mm) of *Xcc* by rhizobacteria
22.	RAC 1	0
23.	RAC 2	0
24.	RAC 3	27.03
25.	RAC 4	0
26.	RAC 5	0
27.	RAC 6	0
28.	RAC 7	0
29.	RAC 8	0
30.	RAC 9	0
31.	Control	0
	SEm±	0.1
	CD at 5%	0.4

carbon source. Maji and Nath (2015) carbohydrates utilization studies shown that *Xcc* grows on medium having dextrose, sucrose, maltose, fructose and lactose as sole carbon source.

Isolation and screening of rhizobacteria against *Xcc*

Dual culture assay technique was used for evaluation of 30 rhizobacteria isolates against *Xcc* under in vitro condition. The Table 3 shows that the rhizobacteria inhibit the growth of *Xcc* by formation inhibition zone around the well at 48 hours after incubation. Among all tested 30 isolates, 3 isolates were able to suppress the growth of *Xcc* (Fig. 3). Isolate RAC 3 developed a maximum inhibition zone of 27.03 mm followed by DHC 5 (19.03 mm) and PUC 5 (12.27 mm). Similarly, Mishra and Arora (2012) also reported that, out of 54 rhizobacteria isolates, 3 isolates viz. TO7, SA3 and CA9 have developed an inhibition zone of 14 mm, 15.60 mm and 21 mm respectively. Liu *et al*. (2016) screened 23 rhizobacteria isolates against *Xcc* and found 18 isolates were effective and developed an inhibition zone ranging between 6mm to 11.67 mm. Naqvi *et al*. (2013) isolated 87 rhizobacteria and out of them, 9 isolates were effective and developed an inhibition zone against *Xcc* under *in vitro*.

ACKNOWLEDGEMENT

I have the proud privilege to express sincere and deep sense of gratitude to the chairman of my advisory committee, Dr. Rajesh Kumar Ranjan and honourable members of my advisory committee member Dr Dinesh Rai, Dr. Md. Abbas Ahmad, Dr. Vipin Kumar RPCAU, Pusa, Samastipur, Bihar. I would like to thank all my colleagues and friends.

I extent my obligatory gratitude to my Godly father Sri Satyanarayan Ji Patidar and mother Smt. Mangi Bai for their endurance, constant encouragement and everlasting blessing throughout my life which acted as a ladder to my success.

Last but not the least I am thankful to Dr. Rajendra Prasad Central Agricultural University, Pusa, Samastipur, Bihar for providing such facilities to accomplish this master degree program.

REFERENCES

Deivamani M, Muthamilan M (2016) Morphological, physiological and biochemical characteristics of *Xanthomonas campestris* pv. *campestris* by black rot of cabbage. *J Innov Agric* 3(1): 10-18.

Elsisi A (2017) Role of antibiosis in control of cabbage black rot caused by *Xanthomonas campestris* pv. *campestris*. *Egypt J Phytopathol* 45(2): 165-181.

Fallahzadeh-Mamaghani V, Golchin S, Shirzad A, Mohammadi H, Mohamadivand F (2021) Characterization of *Paenibacillus polymixa* N179 as a robust and multifunctional bio control agent. *Biol Control* 154: 104-505.

Jensen BD, Vicente JG, Manandhar HK, Roberts SJ (2010) Oc currence and diversity of *Xanthomonas campestris* pv. *campestris* in vegetable *Brassica* fields in Nepal. *Pl Dis* 94(3): 298-305.

Liu K, Garrett C, Fadamiro H, Kloepper JW (2016) Antagonism of black rot in cabbage by mixtures of plant growth-promoting rhizobacteria (PGPR). *Biol Control* 61(5): 605-613.

Lyu D, Backer R, Subramanian S, Smith DL (2020) Phytomicrobiome coordination signals hold potential for climate change-resilient agriculture. *Frontiers Pl Sci* 11: 634.

Maji A, Nath R (2015) A study on pathological aspects of *Xanthomonas campestris* pv. *campestris* causing black rot of cabbage under red lateritic zone of West Bengal. *J Appl Natural Sci* 7(2): 780-785.

Mishra S, Arora NK (2012) Management of black rot in cabbage by rhizospheric *Pseudomonas* species and analysis of 2, 4-diacetylphloroglucinol by qRT-PCR. *Biol Control* 61(1): 32-39.

Naqvi SF, Inam-ul-Haq M, Khan MA, Tahir MI, Ali Z, Rehman HM (2013) Morphological and biochemical characterization of *Xanthomonas campestris* (pammel) dawson pv. sesami and it's management by bacterial antagonists. *Pak J Agric Sci* 50(2): 229-235.

Popović T, Balaž J, Ignjatov M, Mitrović P, Gavrilović V, Jošić D (2014) Identification and genetic characterization of *Xanthomonas campestris* pv. *campestris* as an oilseed rape pathogen in Serbia. *J Pl Pathol* 96(3): 553-560.

Prasanna SL, Ravi S (2014) Isolation and identification of *Xanthomonas campestris* Pv. *campestris* causing black rot on cauliflower from plains of Kerala. *Int J Sci Res* 3(2): In press.

Qiao J, Yu X, Liang X, Liu Y, Borriss R, Liu Y (2017) Addition of plant-growth-promoting *Bacillus subtilis* PTS-394 on tomato rhizosphere has no durable impact on composition of root microbiome. *BMC microbiol* 17(1): 1-12.

Rathaur PS, Singh D, Raghuwanshi R (2016) Characterization and genetic diversity of *Xanthomonas campestris* pv. *campestris* causing black rot disease in crucifers in North India. *Ind Phytopathol* 69(2): 114-118.

Sarıkamış G (2009) Glucosinolates in crucifers and their potential effects against cancer. *Canadian J Pl Sci* 89(5): 953-959.

Schaad NW, Jones JB, Lacy GH (2001) Xanthomonas, In: Schaad NW, Jones JB, Chun W (eds). Laboratory guide for the identification of plant pathogenic bacteria. *Am Phytopathol Soc* 175- 200.

Tistechok S, Skvortsova M, Luzhetskyy A, Fedorenko V, Parnikoza I, Gromyko O (2019) Antagonistic and plant growth promoting properties of actinomycetes from rhizosphere *Deschampsia antarctica* È. Desv.(Galindez Island, Antarctica). *Ukrain Antarctic J* 1: 169-177.

Vicente JG, Conway J, Roberts SJ, Taylor JD (2001) Identification and origin of *Xanthomonas campestris* pv. *campestris* races and related pathovars. *Phytopathology* 91(5): 492-499.

Zhang S, White TL, Martinez MC, McInroy JA, Kloepper JW, Klassen W (2013) Evaluation of plant growth-promoting rhizobacteria for control of Phytophthora blight on squash under greenhouse conditions. *Biol Control* 53(1): 129-135.