RESEARCH ARTICLE

MORPHOLOGICAL IDENTIFICATION AND BIOCHEMICAL CHARACTERIZATION OF *XANTHOMONAS CAMPESTRIS* FROM MUSTARD AND CABBAGE AND ITS MANAGEMENT BY BACTERIAL ANTAGONISTS

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Abstract: Field experiments were conducted during 2018-19 crop seasons to identify resistant genotypes for the management of black rot caused by *Xanthomonas campestris* pv. *campestris.* Morphological and biochemical characteristics of the pathogen were studied .Symptoms of disease were recorded 40-45 days after sowing in different localities of Meerut district of U.P. The optimum temperature for the growth was found 30ºC and white light supported maximum growth of the bacterium. Disease was characterized by the initial symptoms appeared as dark color streaks on the stem from ground level, which girdle the stem making very soft and hollow followed by rotting. Lower leaves showed midrib cracking 'V' shaped yellowing on the leaf margin, browning of veins and weathering. Nutritional studies revealed that sucrose gave maximum growth followed by maltase, 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, mustard, radish and rapeseed. 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: Cabbage, Mustard, Xanthomonas campestris pv. Campestris

INTRODUCTION

In many Asian countries, including India, cabbage (*Brassica oleracea* L. var. *capitata* L.) and other leafy brassicas rank among the most important vegetables. One serious threat to sustainable production of brassicas is black rot disease caused by *Xanthomonas campestris* pv. *Campestris.* Symptoms appeared as irregularly shaped, dull yellow areas along leaf margins which expand to leaf midrib and create a characteristic 'V' shaped lesion; lesions may coalesce along the leaf margin to give plant a scorched appearance. The black rot disease of crucifers is highly destructive, which causes considerable economic damage, 0.03 % infection can lead to epidemics. Infection on crops like crucifers may result in the loss of crop qualitatively and quantitatively. With the progression of disease, infected tissues become black which render normal flow of water and nutrients (Wangeningen and Jan 2005). Strong winds, warm and humid climate also play an important role in the wide spread of the disease (Mugiira et al., 2011). The initial infections may enter from debris of crucifer plants (Mustard, Cabbage and cauliflower etc.), weeds and irrigation water (Roberts et al., 1999).

This disease can be managed by hot water seed treatment for 30 minutes at 50ºC. However, it reduces the seed viability and does not eradicate the pathogen completely (Kishun, 1984). Nursery site should be changed frequently. To reduce secondary spread of the pathogen, Onsando (1988) advocated the use of grass mulch which reduces the extent of splashing of infested soils and hence secondary spread of the pathogen. In India, occurrence of black rot disease is reported from different agro-climatic conditions. The optimum temperature ranges for bacterial growth and host symptom development lie between 25 -30ºC. A slower rate of growth is observed at temperatures as low as 5 and above 35ºC.However, infected hosts are symptomless below 18ºC (Meenu et al., 2013). Generally, this disease is severe under conditions of high rainfall and humidity, with maximum development around 28ºC (Lee and Hong, 2015).

For characterization and identification of plant pathogenic bacteria, classical methods such as symptom and morphological, biochemical and physiological characters are generally used (Schaad et al., 2001).The objective of the present study was under taken to biochemical characterization of Xanthomonas isolates and antibiotic sensitivity profile against Xanthomonas isolates in mustard and cabbage crop and management against phytopathogenic bacteria *Xanthomonas campestris* pv. *Campestris.*

MATERIALS AND METHODS

The experiment was laid out during Rabi 2018-19 at the juridication of Sardar Vallabhbhai Patel University of Agriculture & Technology, Meerut (290 04', N latitude and770 42' „E longitude a height of 237 m above mean sea level), Uttar Pradesh Province, India. The region has a semi-arid sub-tropical climate with an average annual temperature

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of 16.8 0C. The highest mean monthly temperature (38.9 ^{0}C) is recorded in May, and the lowest mean monthly temperature (4.5^{0}C) is recorded in January. The average annual rainfall is about 665 to 726 mm (constituting 44% of pan evaporation) of which about 80% is received during the monsoon period. The predominant soil at the experimental site is classified as Typic Ustochrept. Soil samples for 0–15 cm depth at the site were collected and tested prior to applying treatments and the basic properties were low available nitrogen, low organic carbon, available phosphorus, available potassium medium and alkali in reaction.

Collection of diseased leaf samples

Leaf samples from seed-bed and field plants of cabbage and mustard landraces showing black rot symptoms in the fields, were collected from farms in areas of district Meerut during January and February, 2019 (Bila, 2008). Thirty farms were visited and the incidence of black rot disease in Brassica landraces and cultivars was evaluated. Leaf samples were collected from 20 farms from plants showing black rot symptoms. Samples were placed in paper bags and taken to the laboratory for the isolation of the pathogen.

Identification and Characterization

For identification and characterization of the bacterium, classical methods such as cultural, morphological, biochemical and physiological characteristics were generally used (Schaad et al., 2001). Its cultural, morphological, biochemical and physiological characteristics of bacteria isolated from seed samples and leaf sections were studied by following standard procedures (Pelezar et al., 1957; Dye, 1968; Ryu, 1980). After conducting the various tests, the isolated bacterial strains had identified and characterized by using a reference book (Schaad et al., 2001).

Cultural characteristics

To study cultural characteristics, all the isolated bacteria were grown on nutrient agar, potato dextrose agar, starch agar and yeast extract glucose chalk agar in Petri plates. Inoculated Petri plates were incubated at 28ºC. After three days of incubation, characters of bacterial colonies (color, form, margin, elevation, size and pigmentation) were studied under microscope. The result determined according to Schaad et al. (2001).

Identification characteristics

Gram staining test

Colonies that were grown on YDC agar medium were Gram stained in accordance with the standard Gram staining procedure described by Todar et al. (2005). A thin bacterial smear was prepared from one-day-old culture on glass slide. Then it was air-dried, heat fixed and covered with crystal violet (primary stain) for one minute and was washed in tap water for not more than two seconds. Then Gram's iodine solution was applied for one minute and was washed in tap water. Ethyl alcohol was added drop by drop to wash or decolorize the primary stain and was counterstained with safranin for 30 seconds. Then it was washed in tap water, and was air dried and examined under oil immersion objective lens of a compound microscope for color change, i.e. violet for Gram positive or pink to pink for Gram-negative reaction.

KOH string test

The KOH test was performed by mixing a visible amount of growth from the colony in a loopful (3mm) of 10% KOH on a glass slide. The suspension of bacteria and KOH was mixed continuously with a bacteriological loop. Such a suspension gels become viscous and string out on lifting the loop (positive KOH reaction).

Biochemical characteristics

Citrate utilization test

The Simmon's Citrate medium contains citrate as the sole carbon source and inorganic ammonium salts ($NH_4H_2PO_4$) as the sole source of nitrogen. Bacteria that can grow on this medium produce an enzyme, citrate-permease, capable of converting citrate to pyruvate. Growth is indicative of utilization of citrate, an intermediate metabolite in the Krebs cycle. When the bacteria metabolize citrate, the ammonium salts are broken down to ammonia, which increases alkalinity. The shift in pH turns the bromthymol blue indicator in the medium from green to blue above pH 7.6.

Motility test

Sulphide Indole Motility (SIM) medium is a combination differential medium that tests three different parameters, Sulfur Reduction, Indole Production and Motility. This media has a very soft consistency that allows motile bacteria to migrate readily through them causing cloudiness. The medium contains ferrous ammonium sulfate and sodium thiosulfate, which together serve as indicators for the production of hydrogen sulfide. Hydrogen sulfide production is detected when ferrous sulfide, a black precipitate, is produced as a result of ferrous ammonium sulfate reacting with H_2S gas. Non-motile bacteria generally give growths that are confined to the stab-line, have sharply defined margins and leave the surrounding medium clearly transparent. Motile Bacteria typically give diffuse, hazy growths that spread throughout the medium rendering it slightly opaque.

Catalase test

Catalase mediates the breakdown of hydrogen peroxide H_2O_2 into oxygen and water (it is indicated by bubbles).

Nitrate reduction test

Nitrate reduction test is used for the differentiation of members of Enterobacteriaceae on the basis of their ability to produce nitrate reductase enzyme that hydrolyze nitrate (NO_3^-) to nitrite (NO_2^-) which may then again be degraded to various nitrogen products like nitrogen oxide, nitrous oxide and ammonia

(NH_3) depending on the enzyme system of the organism and the atmosphere in which it is growing.

Triple sugar iron test

0.1% Glucose: If only glucose is fermented, only enough acid is produced to turn the butt yellow. The slant will remain red. 1.0 % lactose/1.0% sucrose: A large amount of acid turns both butt and slant yellow, thus indicating the ability of the culture to ferment either lactose or sucrose. Iron: Ferrous sulfate: Indicator of H_2S formation. If H_2S is produced, the black colour of ferrous sulfide is seen. Some organisms generate gases, which produces bubbles/cracks on the medium. Phenol red: Indicator of acidification (It is yellow in acidic condition and red under alkaline conditions). If neither lactose/sucrose nor glucose is fermented, both the butt and the slant will be red. The slant can become a deeper red-purple (more alkaline) as a result of production of ammonia from the oxidative deamination of amino acids.

5. Obtain the broths from the incubator and observe the colour.

Starch hydrolysis

The Starch Broth medium was used for starch hydrolysis test. The above medium was prepared and sterilized in an autoclave at 15 lbs for 20 minutes and then after cooling poured into four petriplates (20 ml per plate). After solidifying all the four petriplates were inoculated with one loop full test bacterium in the centre of petriplate and incubated at 25±2ºC for 48 hours. Observation for starch hydrolysis was recorded after 48 hours. The plates were flooded with lugol's iodine solution and kept for 10 minutes and colourless zone was observed. The formation of clear zone around the growth of bacterium colony after 10 minutes indicates the hydrolysis of starch and the present of amylase enzyme (Jones and Geider, 2001).

Gelatin hydrolysis

It is used to determine the ability of non-fastidious aerobic organisms to liquefy gelatin by producing gelatinase (proteolytic) enzymes. The principle is that proteolytic enzymes digest proteins and consequently may liquefy gelatin. If the enzymes are present, they attack the gelatin incorporated into the media, resulting in the conversion of the semisolid agar into a liquid. The media were stab inoculated with each isolate grown for 48 hours on gelatin medium and incubated at 28ºC. After 3 and 7 days of incubation, each isolate was evaluated for gelatin liquefaction. The isolates in test tubes were kept in the refrigerator at 4ºC for 30 minutes and gently tipped immediately. A medium that flows readily as the tube is gently tipped was taken as positive for gelatin liquefaction (Kelman and Dickey, 1988).

Antibiogram

Antibiogram of 3 isolates of Xanthomonas from mustard and 1 isolate from cabbage was performed by disc diffusion technique. Different antimicrobial disc(Hi-media) used were Ceftriaxone, Gentamicin, Co-Trimoxazole, Levofloxacin, Netillin, Tetracyclin, Amoxyclav, Ofloxacin(OD278) and Amikacin, Carbenicillin, Co-Trimazine, Kanamycin, Nitrofurantoin, Streptomycin, Tetracycline (OD 007). All the isolates were inoculated in peptone water for 24 hrs at 28ºC. The culture broth of each isolates was spread with the help of cotton swab on the Muller Hinton (MH) agar (Hi-Media) and incubates over night at 28ºC. The plates will be observed for the production of inhibition zone around the octodiscs.

The sensitivity or the resistance of xanthomonas strain to the antibiotic discs was determined by observing the absence or presence of growth around the discs. The zone of inhibition around the colonies was measured to the nearest millimeter. The measurements were compared to manufacturer's instruction for interpretation as sensitive or resistance.

RESULTS AND DISCUSSION

Biochemical characteristics

Different biochemical tests (Triple sugar iron agar, Citrate utilization, Catalase, Oxidase, Nitrate reduction, Urease, Starch hydrolysis, Motility tests etc) were performed for characterization of different isolates. The isolates showed good results in every biochemical tests. Some isolates gave positive results while some gave negative as well. The results of different biochemical tests are represented in Table 1. The isolate that were found to be gram negative rod shaped, obligately aerobic, gram negative, oxidase negative. It was positive for catalase reaction utilized glucose, fructose, sucrose, liquefaction of gelatin and produced hydrogen sulphide and did not produce indole, in addition, the strain failed to reduce nitrate to nitrites Figure 1 & 2. These results is consistent with the finding of Akhtar (1989) who reported that the pathogen causing black rot on cabbage as Xcc based on the biochemical, physiological and pathogenicity characteristics of the isolate from cabbage, which was thought to be the first report of this pathogen on cabbage.

The biochemical characterization showed that out of 29 isolates, 18 isolates were able to hydrolyze starch and all the isolates were positive for catalase test, had the ability to oxidize the glucose by producing high concentration of acid end products. 18 isolates produced H_2S gas in TSI test. The different physiologic and biochemical characteristics such as utilization of glucose, fructose, success for acid production. The isolate hydrolyzed starch gelatin liquefaction, hydrolyzed casein and produced hydrogen sulphide, the strain did not utilize asparagine as sole source of carbon and nitrogen, positive to catalase reaction, did not produce indole and negative to urease and methyl red reaction, did not reduce nitrate to nitrite. These studies confirmed the identify of pathogen (Vinayak, 2012).

Table 1. Biochemical characteristics of *Xanthomonas campestris* causing black rot of mustard and cabbage

S.N.	**Biochemical test**	**Result**	**Number of isolates positive for test**
1.	Gram staining	-	15
2.	KOH Ryu's test	+	15
3.	Citrate test	+	21
4.	Oxidase test	-	14
5.	Catalase test	+	29
6.	Motility test	+	14
7.	Hydrogen sulfide test	+	18
8.	Starch hydrolysis	+	14
9.	Gelatin hydrolysis	+	16
10.	Sucrose fermentation	+	16
11	Fructose fermentation	+	16
12	Glucose fermentation	+	10
13.	Urease test	-	19
14.	Nitrate reduction test	-	18

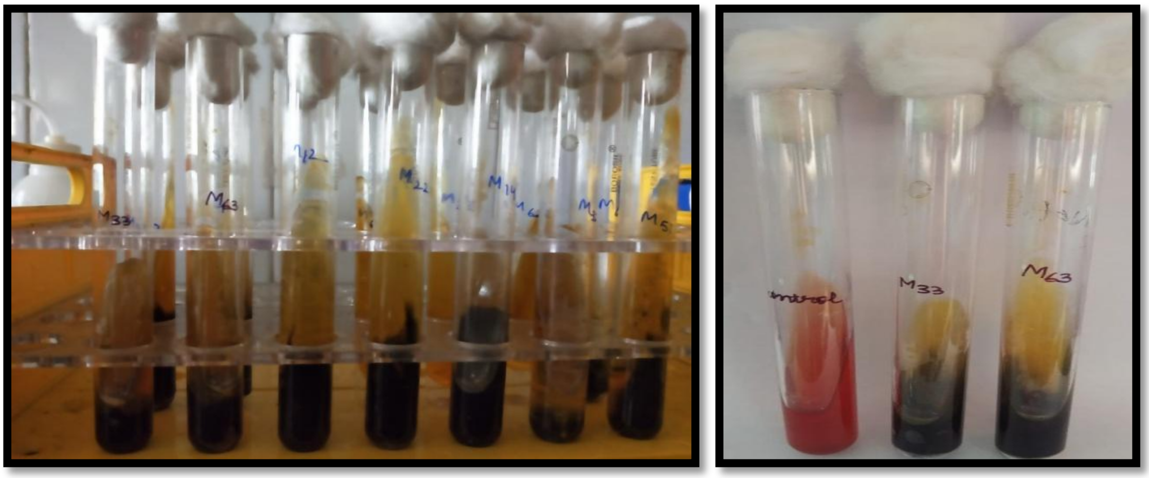

Fig. 1. Test showing utilization of glucose, sucrose, fructose for acid production

Fig. 2. Gelatin liquefaction test for Xanthomonas

Fig. 3. Rapid urease test for Xanthomomas

Different combination of antibiotics against Xanthomonas causing black rot

Different antimicrobial disc(Hi-media) were used Ceftriaxone (30mcg), Gentamicin (10mcg), Co-Trimoxazole (25mcg), Levofloxacin (5mcg), Netillin (30mcg), Tetracyclin (30mcg), Amoxyclav (30mcg), Ofloxacin(5mcg) (OD 278-1PK) and Amikacin(10mcg), Carbenicillin (100mcg), ciprofloxacin (10mcg), Co-Trimazine (25mcg), Kanamycin (30mcg), Nitrofurantoin (300mcg), Streptomycin (10mcg), Tetracycline (30mcg) (OD 007-1PK). All the isolates were inoculated in peptone water for 24 hrs at 28 °C. The culture broth of each isolates was spread with the help of cotton swab on the Muller Hinton (MH) agar (Hi-Media) and incubates over night at 28° C. The plates were observed for the production of inhibition zone around the octodiscs.

Muller Hinton (MH) agar medium making culture plates, the sterile liquid medium was distributed in sterile conical flasks when the temperature cooled down to 40-50ºC. Approximately 15-20 ml of the medium was poured in each petridish. Commercially available and frequently prescribed antibiotics were received as antibiotic discs. Antibiotic disc were placed centrally on the respective plates and incubated at 30 °C for 24 hours.

The present investigation was carried out to evaluate available antibiotic octet to find out their effectiveness against the growth of Xanthomonas under in vitro conditions and results are presented in Table 2 and 3. Among various antibiotics test, ciprofloxacin, gentamicin, levofloxacin produced highest inbition zone of 32 mm, followed by oflaxacin and amikacin of 30 and 26 mm and least effective for co-trimoxazole and nitrofurantoin of 16 mm in diameter. All the isolates were susceptible particularly to gentamicin, co-trimoxazole, levofloxacin, netillin, ofloxacin, amikacin, ciprofloxacin, kanamycin, nitrofurantoin, streptomycin (Figure 4). All of the isolates were resistant,particularly to antibiotics viz., ceftriaxone(Ci), amoxyclav(Ac) and carbenicillin(Cb), co-trimazine. Antibiogram of 4 strains of Xanthomonas, using 2 antiobiotic octodisc revealed varied susceptibility pattern. These studies confirmed

By Chakravarti and Rangarajan (1966) reported that in vitro efficacy of streptocycline on seven isolates of *Xanthomonas*, six isolates of Erwinia and one each of Pseudomonas and Agrobacterium. They found that the chemical was effective against all the pathogens. Whereas, Meena et al. (2007) evaluated the effect of four antibacterial chemicals like Streptocycline, Plantomycin, Copper oxychloride and neem formulations under in vitro disc diffusion technique, and found that Streptocycline was most effective antibacterial chemical against Ralstonia and *Xanthomonas* spp. at 1000 ppm concentration.

Table 2. Zone of inhibition of antibiotics against isolated bacteria (OD 278-1PK)

Antibiotic	Symbol	Disc potency (mcg)	ZOI (mm)	Sensitivity pattern
Ceftriaxone	Ci	30mcg	-	Resistant
Gentamicin	G	10mcg	26.6	Susceptible
Co-Trimoxazole	Co	25mcg	19	Intermediate
Levofloxacin	Le	5mcg	21.2	Susceptible
Netillin	Nt	30mcg	21	Intermediate
Tetracyclin	T	30mcg	19.8	Intermediate
Amoxyclav	Ac	30mcg	-	Resistant
Ofloxacin	Of	5mcg	25.4	Susceptible

Table 3. Zone of inhibition of antibiotics against isolated bacteria (OD 007-1PK)

Antibiotic	Symbol	Disc potency(mcg)	ZOF(mm)	Sensitivity pattern
Amikacin	Ak	10mcg	22	Susceptible
Carbenicillin	Cf	100mcg	32	Susceptible
Co-Trimazine	Cm	25mcg	-	Resistant
Kanamycin	K	30mcg	24.5	Susceptible
Nitrofurantoin	Nf	300mcg	17.5	Intermediate
Streptomycin	S	10mcg	18	Susceptible
Tetracycline	T	30mcg	19	Susceptible
ciprofloxacin	Cb	10mcg	-	Resistant

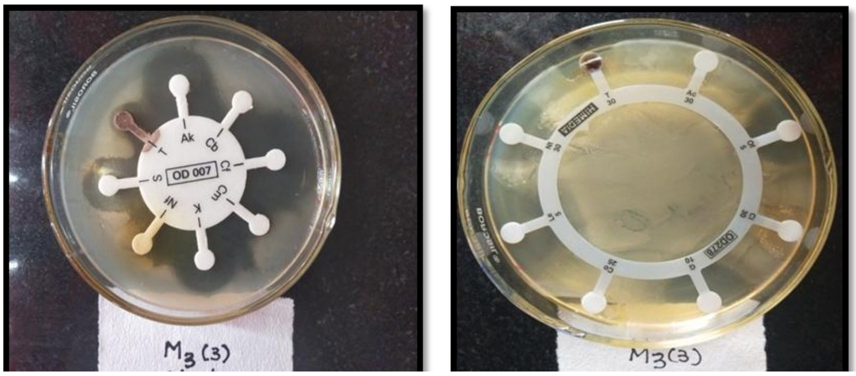

Fig. 4. Antibiotic assay for Xanthomonas

CONCLUSION

It is concluded from the foregoing discussion it is amply clear that pathogen isolated from the black rot disease of mustard and cabbage showed characteristics of the genus Xanthomonas. The morphological characters of several isolates of Xanthomonas campestris were reported that 14 were gram negative straight rods bacteria which show the pink color. Colonies of the isolated bacteria from infected leaves of mustard and cabbage plant were yellow in color on nutrient agar medium. In potassium hydroxide (KOH) test the isolated bacteria showed viscous and form a mucoid string in 15 sec. It also indicates that the bacterium is gram gram-negative. Several biochemical tests such as gram staining, Motility test, Simmons citrate, Urease, Catalase, KOH test, TSI tests, Kovac's oxidase test was done to characterize the Xanthomonas campestris as gram negative bacteria which shows pink color and rod shaped size in staining procedure. The motility test which was accurately confirmed the Xanthomonas campestris motile. The Xanthomonas campestris were able to utilized citrate and the media color change it to blue. The Xanthomonas campestris showed negative result against the urease test which confirmed the bacteria was not able to hydrolyzing urea. The Xanthomonas campestris formed bubbles after added hydrogen peroxide on the top of the bacterial colonies, which was confirmed our bacteria were catalase positive. In TSI test, the gram negative bacteria observed positive results. *Xanthomonas* bacteria turn media red to yellow. The slant and butt yellow color indicates that the bacteria were *Xanthomonas campestris* as it was glucose and lactose fermenting. H_2S was formed, because no black precipitation was found in the medium. Kovac's reagent gives no purple color after 60 seconds, which indicates the Xanthomonas campestris was gram negative bacterium.

Out of 38 isolates 29 showed typical yellow, round, mucoid, small to medium size colony for Xanthomonas on NA medium. These were transferred to starch agar medium, after 24 hrs at 28 $^{\circ}$C bluish green/ bluish white, slimy, round, entire, mucoid colonies were observed. Later the isolates were purified on YDC agar medium. Colonies were straw yellow and bright yellow in color, round, small to medium in size. These isolates were identified, 17 isolates, particularly M12, MM14, M22, M23, M32, M33, M34, M51, M53, M61, M62, M63, M71, M72, C1were Gram negative, rod shaped and positive for Ryu's test. Biochemical characterization was done, out of 14 Gram negative isolates, particularly 10 isolates M12, M14, M32, M33, M35, M51, M53, M63, M64, C1 were positive for *Xanthomonas* spp. On the basis morphological and biochemical results it inferences that the isolate is *Xanthomonas campestris.* For these isolates invitro antibiotic assay was performed. In antibiotic test, ciprofloxacin showed highest of 32mm zone of inhibition, while co-trimoxazole and nitrofurantoin showed the lowest 16mm inhibition zone against *Xanthomonas campestris*. Most of the isolates were resistant, particularly to antibiotics viz., ceftriaxone, amoxyclav, co-trimazine and carbenicillin. So, the antibiotic viz., ciprofloxacin and levofloxacin were found to be the best for control of *Xanthomonas*.

Antibiotic sensitivity test was helpful to find out the control measures of this disease. The zone of inhibition on a plate clearly identified the sensitivity pattern of the isolated bacteria against the different types of antibiotics.

REFERENCES

Chakravarti, B. P. and Rangarajan, M. (1966). Streptocycline an effective antibiotic against bacterial plant pathogens. Hindustan Antibiot. Bull., 8: 209-211.

Kishun, R. (1984). Seed treatment of cabbage black rot. J. Turk. Phytopathol. 13: 81-86.

Lee, Y.H. and Hong, J.K. (2015). Differential defense responses of susceptible and resistant cabbage cultivars to anthracnose, black spot and black rot diseases. Plant Pathology, 64(2): 406-415.

Meena, A. K., Mali, B. L. and Chaudhary, S. L. (2007). Evaluation of partially purified plant products and antimicrobial chemicals preparation against bacterial pathogens. J. Mycol. Pl. Pathol., 37(2): 365-368.

Mugiira, R, B., Arama, P.F., Macharia, J.M. and Gichimu, B.M. (2011). Antimicrobial activity of foliar fertilizer formulations and their effect on ice nucleation activity of *Pseudomonas syringae* pv. *garcae* Van Hall; the causal agent of bacterial blight of coffee. Int J Agri Res. 6:550-561.

Onsando, J.M. (1988). Management of black rot of cabbage caused by Xanthomonas campestris pv. campestris in Kenya. Acta Horticulturae 218: 311-314.

Roberts, S.J., Hiltunen, L.H., Hunter, P.J. and Brough, J. (1999). Transmission from seed to seedling and secondary spread of *Xanthomonas campestris* pv. *campestris* in *Brassica* transplants effect of dose and watering regime. Eur J plant path, 105: 879-889.

Schaad, N.W., Jones, J.B. and Lacy, G.H. (2001). *Xanthomonas*. Laboratory guide for identification of plantpathogenic bacteria, Am Phytopathol SocPress, St. Paul.

Schaad, N.W., Jones, J.B. and Lacy (2001). Gram negative bacteria, *Xanthomonas*. pp. 175-193. In: Laboratory guide for identification of plant pathogenic bacteria. ed. Schaad, N.W., Jones, J.B. and Chun, W. APS Press, St. Paul Minnesota.

Vinayak, N. B. (2012). Investigations on cabbage black rot caused by *Xanthomonas campestris pv. campestris* (Pammel). Master of Science (Agriculture). Thesis. Uni. of agricultural sciences, dharwad – 580 005.

Wangeningen, J.K. and Jan, van der Wolf. (2005). *Alternaria brassicicola* and *Xanthomonas campestris* pv. *campestris* in organic seed production of *Brassica*; Epidemiology and seed infection. Plant Res Int. 363: 01-28.