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CHARACTERIZATION AND VIRULENCE OF *Xanthomonas campestris* pv. *campestris* ISOLATES FROM CRUCIFERACEAE CROPS IN THE NUWARA ELIYA DISTRICT OF SRI LANKA

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ABSTRACT

Black rot disease caused by the bacterium *Xanthomonas campestris* pv. *campestris* (Xcc), has widely distributed resulting in heavy damage to cruciferaceae crops in Nuwara Eliya, Badulla, Kandy and Matale Districts of Sri Lanka in the recent past. . Therefore, a research study was initiated to identify the pathogen using morphological and biochemical characteristics, and the virulence of different isolates. Morphological characteristics of 24 isolates were assessed and 21 isolates were identified as the members of Genus *Xanthomonas*, by using Grams test, Oxidative Fermentative test, H_2S test, Starch hydrolysis and Catalase test. All 21 *Xanthomonas* isolates inoculated on to cabbage (*Brassica oleracea* var. *capitata*) and broccoli (*Brassica oleracea* var. *italica*) were found to be *Xanthomonas campestris* pv. *campestris*. The study of these isolates revealed that four isolates are highly virulent on cabbage and another four isolates are highly virulent on broccoli.

KEYWORDS: Black rot, *Xanthomonas campestris* pv. *campestris, cruciferaceae,* Virulence

INTRODUCTION

Black rot disease caused by *Xanthomonas campestris* pv. *campestris* (Xcc) is one of the most destructive diseases in crucifers worldwide, where yield and quality losses are high (Williams, 1980). The most economically important hosts of *Xanthomonas campestris* pv. *campestris* are the crops belongs to *Brassica oleracea* (*i.e.* cabbage, red cabbage, cauliflower and collards). It also attacks other cultivated crops of Genus *Brassica* and has been reported on a number of cruciferous crops, weeds and ornamentals (Bradbury, 1986).

In Sri Lanka, crucifer crops are cultivated in an extent of 3800 ha annually and the annual production is approximately 37,000 mt (Anon, 2006). Local environmental conditions do not favor the crucifer seed production and hence, Sri Lanka totally depends on imported seeds. Annual crucifer seed requirement is 12.65 mt (Devarrewaere, 1995). Cabbage (*Brassica oleracea* var. *capitata*) is the most important crucifer vegetable grown in Sri Lanka and is cultivated in a large extent mainly in the upcountry. Though black rot disease is reported in Sri Lanka in 1960's (Abeygunawardana, 1969) extensive damage was reported on crucifer crops only recently. Infected seeds are the major source of the pathogen and enable long distance spread of the disease

(Roberts *et al.*, 2007). Muthumala (2006) reported that imported crucifer seeds were heavily infected by the black rot pathogen. There are differences in symptoms of black rot, depending on their host, plant age and environmental conditions (Carissese and Toussaint, 1999). Plants may be affected with black rot at any stage of growth (Williams, 1980) and lead to severe yield losses. Blackening of the veins is resulted by pathogen due to the production of extracellular polysaccharide called Xanthan (Brenner *et al.*, 2005) that eventually plugs the vascular tissue inside the veins causing them to collapse. The tissue above the plug collapses turns yellow, wilts and then the tissue death occurs.

The species *X. campestris* was formerly divided into 123 pathovars according to the host specificity (Bradbury, 1980; Vicente *et al.*, 2001). Recent re-classification of the genus based on DNA-DNA hybridization proposed that the species *Xanthomonas campestris* should be restricted to *Xanthomonas campestris* pv. *campestris* and five pathovars that cause diseases in crucifer plants (Vauterin *et al.*, 1995). In addition, as a result of the distinctions based on the host range (pathovars), several *Xanthomonas* spp. and pathovars have been further differentiated into races based on their interaction with differential cultivars. Kamoun *et al.* (1992) separated the isolates of *Xanthomonas campestris* pv. *campestris* into five different races (0 to 4) based on the response of certain cultivars of turnip (*B. rapa*) and a cultivar of mustard (*B. juncea*). Vicente *et al.* (2001) reported of the existence of six races of *Xanthomonas campestris* pv. *campestris* and twenty non-pathogenic or weakly pathogenic isolates. However, there is a paucity of information available on the occurrence and distribution of the disease and the diversity of the causal organism within Sri Lanka.

Therefore, a study was initiated to isolate the causal organism of black rot from crucifer crops grown in Sri Lanka, establish the identity of the pathogen using morphological and biochemical characteristics, and the virulence of those isolates on different crucifer crops.

MATERIALS AND METHODS

The study was conducted at the Division of Plant Pathology, Agriculture Research Station, Sitha Eliya, Nuwara Eliya, Sri Lanka. Isolation of *Xcc* was carried out using infected leaves with black rot symptoms collected from 10 locations from cabbage (CA: *Brassica oleracea* var. *capitata*), broccoli (BR: *B. oleracea* var. *italica*), red cabbage (RC: *B. oleracea* var. *capitata* f. rubra), collards (CO: *B. oleracea* var. *acephala*), radish (RD: Raphanus sativus) and knol-khol (KK *B. oleracea* var. *gongyloides*) fields in the Nuwara Eliya area. They were placed in clean polythene bags and transported to the laboratory. Samples were rinsed 5 min in running tap water and were prepared according to the method proposed by Ignatov *et al.* (2007), cultured on Potato Sucrose Peptone Agar (PSPA) medium and incubated at 22

±2°C for 48 h in an incubator. Colonies of *Xanthomonas* were picked up based on the colony characteristics *i.e.* color, shape, edge, elevation, odor and opacity. Subsequently, using the characters of colonies *i,e,* Pale yellow, convex, mucous, translucent, thise with starch hydrolyzing zones on PSPA medium after two days of incubation were separated as *Xanthomonas* spp. (Roberts and Koenraddt, 2002). Isolates were named using codes given to each crop (e.g. isolates from cabbage named as CA1, CA2, *etc*).

Typical characteristics of *Xanthomonas campestris* as per Bergey's Manual of Systematic Bacteriology (Brenner *et al.*, 2005) namely aerobic respiration, motility of pathogen, growth above 39°C, presence of mucous growth with 5% glucose, Gram's reaction, H_2S production from peptone, catalase test, and starch hydrolysis were tested using 48 h old cultures according to Dubey and Maheshwari (2004). Virulence of the isolates was studied using cabbage and broccoli plants. Seedlings were raised in plastic pots filled with compost and top soil mixture and they were inoculated four weeks after sowing at three to four true-leaf stage.

Bacterial isolates were grown on Yeast Dextrose Chalk Agar (YDC) medium at 22°C for 48 h before inoculation. Three inoculation points were made on a leaf, using sterile wooden tooth picks. Three leaves were inoculated on a plant and three plants were used for each bacterial isolate separately on cabbage and broccoli. Inoculated plants were kept in a glasshouse for symptoms development during September to October in 2010.

The experiment was arranged in a CRD design with three replicates. Severity of symptoms was assessed by measuring the lesion length for 22 days after inoculation (DAI) at 2 day intervals. Data were statistically analyzed using SAS computer software package and treatment means were separated using DMNRT at 5% probability level.

RESULTS AND DISCUSSION

Morphological characteristics of isolates

Out of 24 isolates, 21 colonies were pale yellow, circular, smooth, convex and mucous with clear zones on PSPA- starch medium after 48 h of growth (Table1).

Typical colony characteristics of Genus *Xanthomonas* are usually yellow, smooth and butyrous or viscid (Buchanan and Gibbbons, 1975) and all 21 isolates gave identical colony characteristics of Genus *Xanthomonas* as defined in the Bergey's Manual of Determinative Bacteria (Bradbury, 2000). However, isolate RD2 from a red radish variety gave creamy yellow, circular, raised colonies while isolate KK1 from knol-khol gave creamy yellow, flat,

circular colonies on PSPA medium. The isolate RD2 from infected leaves of another red radish variety gave circular, smooth, convex colonies but they were dark yellow and less mucous than other isolates obtained from infected crucifer crops.

Table 1. Morphological and biochemical characteristics of different isolates of Xcc

Isolate Nos.	*Source plant*	*Morphological and bio chemical characters*							
		Colony Color	Grams reaction	Mot ility	Growt h at 39°C	Mucoid ity in 5% glucose	Catalase reaction	O/F Test	Starch hydrolysis
CA1[a]	Cabbage	Pale yellow	-	+	+	+	+	O	+
RC2[b]	Red cabbage	Pale yellow	-	+	+	+	+	O	+
BR1[c]	Broccoli	Pale yellow	-	+	+	+	+	O	+
CL1	Cauliflo wer	Pale yellow	-	+	+	+	+	O	+
CO1 [d]	Collard	Pale yellow	-	+	+	+	+	O	+
RD1	Radish	Dark yellow	-	+	_	-	+	F	+
RD2	Radish	Creamy yellow	+	+	_	-	+	F	-
KK1	Knoll-khol	Creamy yellow	-	+	_	-	+	O	_

Note: O – Oxidative F - Fermentative

a - Isolate numbers CA2, C A3, CA4, CA5 and CA6 were from cabbage varieties and showed similar morphological and biochemical characteristics as isolate CA1

b - Isolate numbers RC2and RC3 were from red cabbage varieties and showed similar morphological and biochemical characteristics as isolate RC3

c - Isolate numbers BR2 and BR3 were from broccoli varieties and showed similar morphological and biochemical characteristics as isolate BR1

d - Isolate numbers CO2, CO3, CO4, CO5, CO6, CO7 and CO8 were from collard varieties and showed similar morphological and biochemical characteristics as isolate CO1

The presence of yellow pigments is an important characteristic of Genus *Xanthomonas*. The yellow pigment of 19 members of the genus *Xanthomonas* have been studied as non-water soluble carotenoids (Buchanan and Gibbbs, 1975). Brenner *et al.* (2005) reported that the pigments are highly characteristic 'brominated aryl polyenes' or 'xanthomonadins', and that a

characteristic extracellular acidic heteropolysacharide called 'xanthan' is produced by most strains giving the viscous consistency.

The 21 isolates were motile, Gram negative, Catalase positive and Oxidative bacteria (Table 1). These characteristics are similar to characteristics of Genus *Xanthomonas* as reported in Bergey's Manual of Determinative Bacteriology (Bradbury, 1980). In addition these 21 isolates were able to produce H_2S from peptone, hydrolyze starch, grow at 39°C and present mucodity growth with 5% glucose in Nutrient Agar medium (Table 1). These characteristics are identical to the characteristics of species *Xanthomonas campestris* as reported in Bergey's Manual of Determinative Bacteriology (Bradbury, 1980).

Isolate number RD2 from infected red radish leaves gave positive reaction to Gram's staining and it was fermentative bacteria. Isolate RD1 from another radish variety was Gram negative fermentative bacteria. Genus *Xanthomonas* totally consists of strictly Oxidative Gram negative strait rods (Buchanan and Gibbbons, 1975; Brenner *et al.* 2005). Therefore, isolates RD1 and RD2 do not belong to the Genus *Xanthomonas*. Although isolate KK1 was yellow, Gram negative, and oxidative motile bacteria, it was unable to hydrolyze starch, grow at 39 °C and mucous growth was not present with 5% glucose. Therefore, KK1 may belong to the Genus *Xanthomonas* but not *Xanthomonas campestris*.

Assessment of virulence of isolates

Lesions development in cabbage started 10 DAI and whereas in broccoli it took 8 DAI. In both plants the symptoms of lesions were typical to black rot disease in crucifers, yellowing with blackened veins (Figure 1) and later development of necrosis.

Figure 1. Lesions development in Cabbage (*Brassica oleracea* var. *capitata*) 16 DAI

Isolates RD1 and RD2 did not produce any lesions. But isolate KK1 showed yellowing in 12 DAI without blackened veins and later, a brown color patch was developed around the inoculation point.

The behavior of different isolates on cabbage plant was highly variable. Fifteen isolates produced lesions on cabbage plants 10 and lessons were visible twelve days after inoculation in the other six isolates. (Table 2).

Initially isolate CA2 showed longest lesion length and isolates CO8 developed the longest lesion 14 DAI and continue to grow up to 18 DAI (Table 2). The isolates CO5, CO3 and BR2 produced the highest lesions from 16 DAI and produced the longest lesions 31.54 mm and 31.58 mm 22 DAI (Table 2). However, the isolate BR3 initially produced small lesions but grew into the longest lesions on the 22nd DAI (Table 2). The smallest lesion size was recorded in the isolates RC1 & CA3.

Development of lesions on Broccoli was different from cabbage (Table 3). All the isolates produced typical black rot lesions on broccoli seedlings.

Lesions were observed in twelve isolates 10 DAI and in the other nine in 12 DAI. Initially RC1 showed the longest lesion, later 12 DAI CO8, BR2 and CO4 exceeded RC1. Finally, CO4, RC3, CA2 and CO6 produced the longest lesion at 22 DAI. The least virulent isolate was identified as CA4.

In cabbage and broccoli typical symptoms of black rot were observed, but the rate of lesion development by different *Xcc* isolates varied highly. The variation in virulence of the isolates may be due to pathogenic factors and the resistance exerted by the host plant to the pathogen. Michael *et al.* (1984) proposed that pectolytic enzymes, xanthan and low molecular weight acidic phytotoxic substances are involved in the development of disease symptoms due to the infection of *X. campestris* pathovars.

The pathogenicity locus within *Xcc* comprises two genes governing the biosynthesis of lipopolysaccharide within the bacterial cell (Brenner *et al.*, 2005). Lipopolysaccharide protects the bacteria against antibacterial substances produced by the plant early in the infection process. The amount of lipopolysaccharide produced by different isolates may vary and thus, the time taken to develop symptoms and rate of symptom development also vary considerably (Michael *et al.*, 1984). Further, research has proven that certain enzymes involved in pectin and polygalacturonate degradation play a limited role in pathogenesis (Brenner *et al.*, 2005).

Table 2. Average lesion diameter (mm) of different isolates in cabbage

Isolate No.	*Lesion diameter (mm)*						
	10 DAI	**12 DAI**	**14 DAI**	**16 DAI**	**18 DAI**	**20 DAI**	**22 DAI**
CO1	1.25 cbd	7.33 ba	13.83 bac	21.24 ba	24.65 bdac	25.66 bac	26.25 ba
CO2	0.50 cd	3.19 ecd	8.56 egdfch	14.31 ebdfc	18.12 ebdgcf	19.69 ebdac	20.75 ba
CO3	0.00 d	4.58 bcd	15.08 ab	23.54 a	26.00 ab	30.04 a	31.54 a
CO4	0.00 d	3.25 ecd	7.94 egdfh	21.24 ba	21.87 edac	25.83 bac	27.94 ba
CO5	0.00 d	4.29 bcd	9.08 egdfch	23.08 a	27.67 a	30.42 a	31.58 a
CO6	0.70 cbd	2.40 ecd	6.00 egfih	10.95 edf	16.60 edgcf	21.45 bdac	24.30 ba
CO7	2.85 a b c	3.60 becd	6.81 egfh	12.37 edfc	16.60 ebdgcf	25.06 bdac	27.81 ba
CO8	2.69 cbd	4.75 bcd	17.00 a	24.600 a	28.050 a	29.05 ba	29.05 ba
BR1	1.37 cbd	3.41 ecd	9.45 egdfc	19.65 bac	25.20 abc	39.30 a	31.50 a
BR2	0.50 cd	4.00 bcd	11.83 ebdac	24.50 a	28.25 a	29.25 a	29.75 ba
BR3	2.75 abd	3.85 bcd	7.75 egfh	12.39 edfc	21.62 ebdacf	28.50 ba	33.00 a
RC1	0.00 d	5.58 bc	10.33 ebdfc	13.75 ebdfc	15.49 edgf	24.30 bdac	17.53 b
RC2	0.50 cd	1.30 ed	3.70 gih	8.50 egf	13.80 ehgf	23.37 bdac	25.75 ba
RC3	0.54 cd	2.87 ecd	3.25 ih	7.85 gf	16.20 edgcf	22.94 bdac	27.14 ba
CA1	1.00 cbd	1.50 ed	3.65 gih	7.60 gf	12.35 hgf	17.85 ebdac	20.90 ba
CA2	5.75 a	9.000 a	13.50 bdac	17.81 bdac	19.80 ebdagcf	22.19 bdac	22.85 ba
CA3	2.00 cbd	3.62 becd	4.47 gfih	7.94 gf	11.50 hg	14.06 ed	17.44 b
CA4	2.75 cbd	3.90 bcd	6.0 egfih	13.85 ebdfc	18.05 ebdgcf	21.80 bdac	25.20 ba
CA5	2.20 cbd	3.85 bcd	4.75 gfih	11.95 edfc	16.75 edgcf	22.15 bdac	26.05 ba
CA6	3.37 b	4.25 bcd	7.26 egfh	12.39 edfc	16.32 edgcf	29.05 ba	29.12 ba
CL1	0.00 d	0.000 e	1.000 i	1.37 g	5.37 h	9.625 e	22.37 ba
CV%	83.52	46.25	42.84	34.15	29.12	28.23	29.03

Within each column values followed by same letter are not significantly different at p=0.05.
DAI – days after inoculation

Table 3. Average lesion diameter (mm) of different *Xcc* isolates in broccoli

Isolate No	*Lesion diameter (mm)*						
	8 dai	10 dai	12 dai	14 dai	16 dai	18 dai	20 dai
CO1	0.00 ^{b}	2.39^{edgf}	7.64^{bdac}	15.19^{ebdac}	19.64^{edbagcf}	22.62^{ebdac}	23.75^{bdac}
CO2	0.00 ^{b}	2.21^{edgf}	9.35^{bac}	13.90^{ebdc}	19.08^{ebdagcf}	22.31^{ebdac}	24.25^{bdac}
CO3	0.55 ^{b}	7.51^{ba}	11.58^{ba}	17.17^{bac}	19.66^{ebdacgf}	20.67^{ebdac}	21.67^{ebdac}
CO4	0.17 ^{b}	3.21^{ebdgcf}	12.33^{a}	15.92^{bdac}	25.17^{a}	29.00^{a}	29.00^{a}
CO5	0.00^{b}	3.04^{edgcf}	7.44^{bdac}	10.25^{edc}	13.50^{gf}	15.50^{ed}	16.81^{ebdc}
CO6	0.56 ^{b}	5.08^{bdac}	10.87^{ba}	16.38^{bac}	22.24^{bac}	25.48^{ba}	26.15^{a}
CO7	0.00 ^{b}	0.42^{gf}	3.43^{dc}	9.04^{ed}	14.50^{edgf}	19.25^{ebdc}	22.50^{ebdac}
CO8	0.00 ^{b}	7.12^{bac}	14.06^{a}	21.37^{a}	23.41^{ba}	25.12^{ba}	25.12^{bac}
BR1	0.00 ^{b}	2.00^{edgf}	5.46^{bdc}	12.25^{edc}	15.21^{edgcf}	15.75^{ed}	16.33^{ebc}
BR2	0.44 ^{b}	4.71^{ebdacf}	13.31^{a}	19.75^{ba}	23.12^{bac}	24.56^{bac}	25.69^{ba}
BR3	0.00 ^{b}	0.00 ^{g}	2.083^{d}	9.92^{edc}	12.58^{g}	16.41^{edc}	15.74^{ed}
RC1	3.37 ^{a}	8.33^{a}	11.918^{ba}	15.67^{ebdac}	20.91^{ebdacf}	22.668^{bdac}	22.67^{ebdac}
RC2	0.33 ^{b}	1.83^{edgf}	7.293^{bdac}	10.52^{edc}	16.90^{ebdagcf}	21.063^{ebdac}	22.81^{ebdac}
RC3	0.00 ^{b}	1.12^{egf}	5.250^{bdc}	12.44^{edc}	18.37^{ebdagcf}	21.875^{ebdac}	25.87^{a}
CA1	0.81 ^{b}	4.56^{ebdacf}	10.188^{ba}	16.12^{bac}	20.67^{ebdacf}	23.313^{bdac}	23.56^{bdac}
CA2	0.37 ^{b}	5.06^{ebdac}	10.813^{ba}	14.81^{ebdac}	21.81^{ebdac}	25.375^{ba}	26.25^{a}
CA3	0.87 ^{b}	6.25^{bdac}	10.125^{ba}	13.00^{ebdc}	15.37^{edgcf}	19.375^{ebdc}	20.50^{ebdac}
CA4	0.44 ^{b}	6.33^{bdac}	10.333^{ba}	12.44^{edc}	13.94^{egf}	14.600^{e}	14.61^{e}
CA5	0.00 ^{b}	3.62^{ebdgcf}	7.738^{bdac}	12.56^{edc}	20.94^{ebdacf}	24.125^{bac}	24.81^{bac}
CA6	0.31 ^{b}	2.80^{egdcf}	9.375^{bac}	13.39^{ebdc}	20.61^{ebdacf}	23.425^{bdac}	24.24^{bdac}
CL1	0.37 ^{b}	2.56^{edgf}	8.570^{bdac}	11.44^{edc}	19.31^{ebdagcf}	22.000^{ebdac}	23.56^{bdac}
CV%	142.32	27.19	33.49	30.61	24.36	23.24	22.99

Within each column values followed by same letter are not significantly different at p=0.05.
DAI – days after inoculation

Intra-molecular interaction of gum constituents could also play a significant role in virulence of Xcc (Brenner *et al.*, 2005). The constituents of Xanthan gum produced by different isolates obtained from different host plants in this study may have varied and could be a reason for the varying

degree of virulence reported. The other factor that may play an important role in virulence of a pathogenic bacterium is the ability to acquire nutrients from the host to establish an infection. The *X. campestris* pv. *campestris* propagates and spreads in the apoplast of the host plant after infection. Thus, the ability to acquire nutrients from the apoplast is critically important for it to cause disease (Brenner *et al.*, 2005). However, the nutritional requirements of *X. campestris* pv. campestris during infection and the molecular mechanism by which it acquires nutrients from the apoplast are still unclear (Dong *et al*, 2005). If the nutritional requirement and the molecular mechanism by which it acquires nutrients could be resolved, differences in virulence of different isolates can be explained. The other factors influence the extent of symptoms development of *Xcc* and type of symptoms expressed are environmental conditions under which the plant grows and the cultivar of the host plant (Williams, 1980). However, the mechanisms involved in expression of symptoms are not investigated thoroughly (Brenner *et al.*, 2005).

CONCLUSIONS

This study revealed that several isolates of *Xcc* are present in main cruciferous crops growing in Nuwara Eliya of Sri Lanka. Further, 21 isolates belonging to the Genus *Xanthomonas* were identified. Different levels of virulence of these isolates were also recorded on cabbage and broccoli. The recent outbreak of black rot in Sri Lanka could be due to the presence of variable populations of Xcc. Isolates used in this study are being maintained in the laboratory and could be used for future studies. These cultures can be deposited at a culture collecting center for future references.

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