Occurrence and Diversity of *Xanthomonas campestris* pv. *campestris* in Vegetable Brassica Fields in Nepal

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ABSTRACT

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Black rot caused by *Xanthomonas campestris* pv. *campestris* (*Xcc*) was found in 28 sampled cabbage fields in five major cabbage growing districts in Nepal in 2001, and in four cauliflower fields in two districts and a leaf mustard seed bed in 2003. Pathogenic *Xcc* strains were obtained from 39 cabbage plants, four cauliflower plants and one leaf mustard plant with typical lesions. Repetitive DNA polymerase chain reaction-based fingerprinting (rep-PCR) using REP, ERIC and BOX primers, was used to assess the genetic diversity. Strains were also race-typed using a differential series of *Brassica* spp.. Cabbage strains belonged to five races (1, 4, 5, 6 and 7) with races 4, 1 and 6 the most common. All cauliflower strains were race 4, and the leaf mustard strain was race 6. A dendrogram derived from the combined rep-PCR profiles showed that the Nepalese *Xcc* strains clustered separately from other *Xanthomonas* species and pathovars. Race 1 strains clustered together and strains of races 4, 5 and 6 were each split into at least two clusters. The presence of different races and the genetic variability of the pathogen should be considered when resistant cultivars are bred and introduced into regions in Nepal to control black rot of brassicas.

Additional keywords: genetic variation, pathogenicity

INTRODUCTION

Cabbage and cauliflower production increased significantly in Nepal during the 1990's; in 2004/2005, nearly 20,000 ha were cultivated with cabbage, accounting for 11% of the vegetable area in Nepal (3). Cabbage is considered an important cash crop, and in some areas production is almost continuous, with up to three crops per year. The production increase has been accompanied by an increase in the use of commercial hybrid seed (32).

Black rot, caused by the vascular bacterium *Xanthomonas campestris* pv. *campestris*, (*Xcc*), is one of the most serious diseases of vegetable Brassicas (4, 43). Typical disease symptoms in the field are V-shaped, chlorotic to necrotic lesions starting from leaf margins and with blackening of the vascular tissue (33, 43). Blight symptoms have also been reported (45). Black rot of crucifers was reported in Nepal in 1977 (30). Adhikari & Basnyat (1) found that 20 strains of *Xcc* from broccoli, cabbage and cauliflower from the Kathmandu and Chitwan valleys, had similar biochemical and physiological characteristics, but strains from Chitwan tended to be more aggressive on cabbage, cauliflower and broccoli, than strains from the Kathmandu Valley.

Until 2007, six races of *Xcc* were defined on the basis of their reactions on a differential set that includes genotypes of *Brassica oleracea*, *B. rapa*, *B. juncea*, *B. napus* and *B. carinata* (14, 40). A gene-for-gene model has been proposed to explain the interactions between the races and the differential cultivars (40). Races 1, 4 and 6 are the most frequently reported races (11, 39, 40). In 2007, race 7 was reported in Belgium by Fargier & Manceau (8), along with races 8 and 9, which had a narrower host range on differential

cultivars. We also found race 7 in this study and a preliminary report of our work appeared in 2007 (13).

Conserved, repetitive DNA sequences in bacteria have been targeted for use in distinguishing bacterial species and strains by the polymerase chain reaction (PCR) (7, 37, 38), using primers specific to the repetitive extragenic palindromic (REP) sequence (10), the enterobacterial repetitive intergenic consensus (ERIC) sequence (28), and the BOX element (17). Previous studies have shown the potential of repetitive DNA PCR-based fingerprinting (rep-PCR) to differentiate *Xanthomonas* pathovars (20, 21, 26, 41) and strains within pathovars (16, 20), and therefore could be used as a diagnostic tool in plant pathology.

Some strains of *Xcc* have been characterised by genetic fingerprinting using rep-PCR. *Xcc* strains from Tanzania tended to cluster within local geographical areas (18) and similarly it was found that *Xcc* strains from different countries to some extent clustered within larger geographical regions (35), whereas this was not the case in a study carried out on Italian strains (46). Results of rep-PCR fingerprinting allowed the separation of *Xcc* from *X. campestris* pv. *raphani* (*Xcr*) strains, and showed that among *Xcc* and *Xcr* strains there was a tendency for strains of the same race to cluster together (41). Rep-PCR, AFLP, PFGE (pulsed-field gel electrophoresis) and integron gene cassette PCR was used to study the diversity of 22 *Xcc* strains, and revealed 11, 12, 13, and 4 differentiated genotypes, respectively, supporting the heterogeneous nature and relatively high level of diversity in this pathovar (36, 46).

The aim of this work was: (i) to study the occurrence of black rot in Brassica fields in major cabbage growing regions of Nepal; (ii) to characterise the diversity of the *Xcc*

strains by rep-PCR fingerprinting, and race typing on a set of differential *Brassica* spp. genotypes with the aim to suggest efficient control strategies involving breeding, introduction and cultivation of resistant cultivars.

MATERIALS AND METHODS

The work was divided into three steps: 1) Collection of black rot infected leaf material in Nepal, 2) isolation of *Xcc* and pathogenicity testing at the Faculty of Life Sciences, Denmark, and 3) further pathogenicity testing, rep-PCR and race typing at Warwick HRI, The University of Warwick, UK.

Collection of black rot infected leaf samples. Twenty-eight white cabbage (*Brassica oleracea* var. *capitata*) fields, each at least 10 x 10 m, were sampled in five major cabbage growing areas (five districts in four zones) in the Eastern, Central and Western parts of Nepal (Fig. 1; Table 1) during the monsoon season in July 2001. According to farmers' information, the cultivar grown was usually Green Coronet F_1. From each field, a leaf with typical black rot symptoms (V-shaped lesions) was sampled from each of ten well-separated plants. In 2003, single plants in four cauliflower (*B. oleracea* var. *botrytis*) fields were sampled, and a single sample was collected from a seedbed of leaf mustard (*B. juncea*). Samples were dried between sheets of paper at room temperature before isolation of the causal agent. The altitude of each field was recorded using a hand held GPS device (eTrex Venture GPS navigator, Garmin Corporation, Taiwan).

Isolations. One or two leaf samples, representing one or two plants from each field, were selected for isolation of the causal agent from the typical V-shaped lesions. From each leaf sample, 0.5-1 cm^2 leaf tissue was excised with a sterile scalpel from the margin of a

lesion and placed in a drop of sterile 0.85% saline for 5 min. A loopful of saline suspension from a point with bacterial ooze was streaked on nutrient starch cycloheximide agar (NSCA) (23) and incubated at 28°C for 48-72 h and observed for typical *Xcc* colonies (pale yellow, mucoid, starch-hydrolyzing). A typical colony from each isolation plate was sub-cultured to yeast dextrose calcium carbonate agar (YDC) (31), and then transferred to sucrose-peptone agar (SPA) for viscosity testing (19). For most isolations, and additional colony from the same plate was sub-cultured and the strain was kept as a 'duplicate'. All isolates with a flow time of more than 30 seconds (19) were subsequently tested for pathogenicity. Isolates were stored at -80°C in cryovials (Simport cryovial, Canada) containing glass beads and nutrient broth plus 15 % glycerol (9), or in Protect cryovials (Technical Service Consultants Ltd., Lancashire, UK).

Pathogenicity. Isolates were tested for pathogenicity on six 3 to 4-week-old plants of cabbage cv. Copenhagen Market (L. Daehnfeldt, Denmark), grown in 12 x 13 cm square pots in a peat-based compost (Weibulls Enhetsjord, K-Jord, Svalöf Weibull Trädgärd AB, Hammenhög, Sweden) in a glasshouse at 20-25°C. Each isolate was tested twice (different inoculation date). Bacteria were grown on YDC agar at 28°C for 48 h, and inoculum was prepared by suspending a small amount of growth in 0.85% saline. The suspensions were adjusted to a final concentration of approx.10^8 CFU ml^{-1} by dilution. Each pot with six plants was sprayed with 10 ml of bacterial suspension using a hand held plastic atomiser (KAB1005, KABI, Denmark) at a distance of approx. 15 cm from the plants. After inoculation, plants were maintained in a transparent polyethylene tent for 48 h to increase the humidity. The temperature varied between 25 and 35°C during the

experiment with a day length of approx. 16 h. The six plants were assessed for symptoms (V-shaped lesions) 2 and 3 weeks after inoculation; isolates were considered pathogenic when V-shaped lesions occurred on at least three plants. In addition, the pathogenicity of twenty isolates was tested on individual 4-week-old Savoy cabbage plants (*B. oleracea* var. *sabauda*) of cv. Wirosa F_1 (Bejo Zaaden, The Netherlands) using the inoculation method of Vicente et al. (41). Plants were raised from seed sown in 6.5-cm square plastic pots with Levington M2 compost (Scotts, England) in a glasshouse with a minimum temperature of 20/15°C and venting at 22/17°C (day/night), and with supplementary lighting to give a 16 h photoperiod. Isolates were grown on YDC medium for 48 h at 30°C. The three youngest leaves of 4-week-old plants were inoculated at six sites near the leaf margin and three sites in the midrib using a sterile pin charged with growth from a YDC plate (41). One plant was inoculated per isolate, but the inoculations were repeated if the first results indicated that the isolate was weak or not pathogenic. The presence of symptoms was recorded two and three weeks after inoculation (40).

rep-PCR fingerprinting. DNA extraction and rep-PCR amplification was done for all pathogenic strains from Nepal (44 independent strains plus 32 duplicate strains from the same leaf) and two reference strains, representing two commonly found races, HRI 1279A (race 4) and HRI 3811 (race 1), under the same laboratory conditions at Warwick HRI as previously reported (41).

All samples, 'blank' control lanes using water as template, and the two reference strains were included at the same time in each of the rep-PCR setups. From each strain and blank controls, 6 µl aliquots (REP, and ERIC primer sets, and 2.5 µl aliquots (BOX primer) of amplified PCR products were separated by electrophoresis in 1.2% agarose 22 lane gels

(41). A molecular mass marker (1-Kb Plus DNA Ladder, Invitrogen) was loaded on both sides and central lanes, and the two reference strains were loaded on all gels. Gels were visualized on a UV transilluminator and photographed on an imaging system (ImaGo, B & L Systems, The Netherlands). A digital image of each gel was analysed using the GelCompar II Software (Applied Maths, Kortrijk, Belgium). Gels were normalised using the molecular mass markers; optimisation settings were chosen which maximised the similarity of the strains, which were repeated on each gel. Bands less than 500 bp (REP), 400 bp (ERIC), 300 bp (BOX) and larger than 4000 bp were excluded from the analysis. The normalized data generated from BOX, ERIC and REP fingerprinting profiles were combined. The similarity between profiles was calculated using the Pearson product-moment correlation coefficient. Cluster analysis of the pairwise similarity values was performed using the UPGMA algorithm. For comparative purposes, profiles of additional strains, representing all six races of *Xcc*, three races of *Xcr*, three strains of other *Xanthomonas campestris* pathovars and two strains of other *Xanthomonas* species generated previously under the same laboratory conditions as used here (41) were included in the analysis.

Race typing. Forty-four independent strains from Nepal and seven duplicate strains (obtained from the same plants), were race typed. Race typing was carried out using a set of differential *Brassica* spp. using the method and experimental conditions at Warwick HRI as previously described (40) with the following modifications: the doubled haploid lines Cob60 and FBLM2 and the inbred line Bo99075 derived respectively from *B. napus* cv. Cobra line 14R, *B. juncea* cv. Florida Broad Leaf Mustard and *B. carinata* accession PI 199947 replaced the lines from which they were originally derived. In addition, the

doubled-haploid line SxD1 derived from a cross between *B. oleracea* cv. Böhmerwaldkohl and a rapid cycling line was also tested to confirm the results obtained with Miracle F_1. The differentials used are included in Table 2. Three plants of 'Seven Top Turnip' and one plant of each of the other genotypes were inoculated with each strain. One control strain of race 5 (HRI 3880) was also race typed for comparison.

Plants were raised in the glasshouse as described above for the pathogenicity test on Wirosa F_1. Strains were grown on YDC medium for 48 h at 30°C, and suspended in 0.85% saline to produce a turbid suspension containing approx. 10^8-10^9 CFU ml^{-1}. The three youngest leaves of 4-week-old plants were inoculated by clipping the veins near the leaf margins with mouse-tooth forceps with teeth wrapped in absorbent cotton wool and dipped in the bacterial suspension (40). Disease assessments were carried out two and three weeks after inoculation (40).

RESULTS

Occurrence of black rot and symptoms in the field. Black rot symptoms were commonly observed in all 28 cabbage fields visited and sampled in the five major cabbage growing districts in Nepal during the monsoon season in July 2001 (Fig. 1, Table 1). The altitude of the fields ranged from 1296 to 2139 m. Most samples were collected from fields in Lalitpur, Dhankuta, and Makawanpur districts, as cabbage production was at its peak at the time of sampling, whereas most fields had already been harvested in Bhaktapur and Baglung districts. Typical V-shaped, chlorotic lesions with black veins were found on leaves in all fields, irrespective of plant maturity. In some fields, mature heads exhibited blackening of the vascular stem tissue when cut in half. In 2003, the four

cauliflower fields and the seedbed of leaf mustard visited, also exhibited typical symptoms: V-shaped, chlorotic lesions with dark veins. Blight symptoms were not observed.

Isolations and pathogenicity. Isolates from 39 cabbage plants, four cauliflower plants and one leaf mustard plant produced yellow mucoid growth on YDC and a positive viscosity test (Table 1, Table 3). Thirty duplicate isolates from the same cabbage plants and two duplicate isolates from the cauliflower plants also had the above typical characteristics. The isolates all produced clear and distinct V-shaped lesions on leaves of *B. oleracea* cv. Copenhagen Market and/or Wirosa F_1 and were considered to be *Xcc.*

rep-PCR fingerprinting. All 39 cabbage strains, the four cauliflower strains and the leaf mustard strain together with the 32 duplicate strains from Nepal and the two reference strains, HRI 1279A (race 4) and HRI 3811 (race 1), were fingerprinted using rep-PCR (Table 1). The rep-PCR fingerprint profiles, obtained with the BOX, ERIC and REP primers, confirmed that all isolates were strains of *Xcc*. These profiles were complex and revealed polymorphic bands among *Xcc* strains. The combined dendrogram had a cophenetic correlation coefficient of 0.95. The separate dendrograms obtained with REP, ERIC and BOX had cophenetic correlations of 0.93, 0.94 and 0.88 respectively (data not shown).

The profiles of the two reference strains (HRI 1279A and 3811) that were repeated in five different gels had similarities ranging from 88.6 to 97.7% and 81.1 (in one gel) to 95.3% respectively, with most profiles of the same strain having similarities greater than 93%. Thus, the separation of strains with similarities greater than 80% might not be reliable, and strains with a similarity greater than approximately 90% might be identical.

All, except one, of the pairs of strains originating from a single lesion (30 pairs from cabbage, two from cauliflower plants), produced fingerprint profiles that were identical (i.e. with a similarity ≥ 90%). The pair of strains (N47 and N48) obtained from the same lesion had distinct profiles (similarity of 81.9%, with profiles obtained on the same gel runs).

Figure 2 presents a dendrogram constructed with the results from Nepalese strains from 39 individual cabbage plants, of which one plant is represented by two strains (N47 and N48), four strains from cauliflower, one strain from leaf mustard, fifteen strains representative of the six races of *Xcc*, and eight strains of other *Xanthomonas* species and pathovars. All *Xcc* strains from Nepal and all reference *Xcc* strains, except race 2, formed a single cluster at 44.8% similarity. This group was differentiated from the other *X. campestris* pathovars and other *Xanthomonas* species. *Xcc* race 2, of which only a single strain has been obtained (39), is clearly distinct from other *Xcc* strains. At 70% similarity, the *Xcc* strains separated into a large group of 29 strains (containing 21 strains from four districts of Nepal), a group of 12 Nepalese strains (from four districts of Nepal), a group of 11 strains (containing 9 Nepalese strains from three districts of Nepal), two strains from Nepal (N68 and N70), two strains from Portugal and Nepal (HRI 6185 and N8), respectively, and three individual strains (HRI 6181, HRI 3880, HRI 6312A). At 80% similarity, the group of 29 strains split into two major groups (containing 9 and 13 strains), three minor groups containing two strains and an individual strain (HRI 6412); the group of 12 strains split into a group of 8 strains, a group of two strains (N35 and N47) and two single strains (N55 and N34); the group of 11 strains remained clustered.

Race-typing. Thirty-nine cabbage *Xcc* strains, seven duplicate strains, four cauliflower strains, and one leaf mustard strain from Nepal plus the race 5 type-strain (HRI 3880) were inoculated into the differential set (Table 2). Four of the six previously described races, namely 1, 4, 5, and 6 were identified (Table 3; Table 4). In cabbage, on an individual plant basis, 14 strains were identified as race 4, 12 strains as race 1, nine strains as race 6, and three as race 5. Races 2 and 3 were not found. Race 6 strains were generally pathogenic on all differentials, but weakly pathogenic on *B. oleracea* cv. Miracle F_1 and line SxD1. Two strains, N35 and N47, were pathogenic and very aggressive on all genotypes except on *B. juncea* line FBLM2. This was an unknown differential reaction at the time of the experiments, and these strains were assigned to race 7 (13). Seven plants were represented by seven pairs (duplicates) of strains from the same lesion (Table 3). These pairs of strains were the same race-type, except for one pair (N47 and N48), which were identified as races 6 and 7, respectively (Table 3). Races 4, 1 and 6 were the most common in cabbage and were all found in four of the five districts (Table 4). Races 5 and 7 were represented by three and two of the strains, respectively (Table 4). Race 5 was identified in two of the districts, and race 7 in only one district. The four strains from cauliflower were all race 4, and the leaf mustard strain was race 6 (Table 3, Table 4). Cabbage production in the Dhankuta district was intensive at the time of sampling, and all five races identified in this study were present. Three races were identified amongst the 13 strains from Lalitpur (Table 4) whereas 2 or 3 races were identified on the basis of a more limited number of strains in the other districts. More than one race was present in 11 out of 17 tested cabbage fields, on the basis of tests of strains from two to three plants per field (Table 3).

Typical black rot symptoms were frequent on inoculated plants, and dark necrotic, blight symptoms were observed in some cases. Blight-like symptoms were frequently produced by race 7 strains and by some strains of races 5 and 6, and a few strains of race 1, but not race 4 strains.

Combining rep-PCR fingerprinting, race affiliation and geographical origin. The geographical origin of the Nepalese strains does not seem to be correlated with the rep-PCR clusters shown in the combined dendrogram (Fig. 2). The clusters seem to be more related to the race of the strains. All 12 Nepalese *Xcc* race 1 strains formed a single sub-cluster at 85% similarity jointly with the race 1 reference strain (HRI 3811) from USA. The *Xcc* race 4 strains were divided amongst two distinct clusters, containing nine and 11 strains, at 81% and 85% similarity, respectively. The latter group included the race 4 reference strain (HRI 1279A) with rep-PCR profiles from the present study and a race 4 strain (HRI 6189) from a previous study (40, 41). Strains from the Lalitpur district were included in both race 4 clusters, even at the field level (strains N20/21 and N27). Most of the race 1 strains and strains in each of the race 4 clusters might be identical. Two Nepalese race 5 strains (N68 and N70) were identical and distinct from another race 5 Nepalese strain (N34). Nine race 6 and the two race 7 Nepalese *Xcc* cabbage strains were included in the same cluster at 77% similarity. One Nepalese race 6 strain (N8) clustered with the race 6 reference strain from Portugal.

DISCUSSION

The present study supports previous findings related to the occurrence of black rot in Nepal and the diversity of the causal bacterium, *Xcc* (1, 27), and it confirms that the disease is widespread in the vegetable Brassica growing areas in the country. Symptoms of black rot were observed in all visited fields, in seedbeds, in newly transplanted crops and in mature crops. The disease is frequently overlooked by farmers and extension workers, who observe symptoms, but mistakenly think they are related to natural plant senescence. The increased and almost continuous production of Brassica crops in some areas, the ability of *Xcc* to spread by seed movement (42) and rain splash (43) and to survive in plant debris left in the field (24), may cause build up and maintenance of inoculum, thereby increasing the impact of the disease in the future, unless appropriate control measures are adopted.

A significant proportion of cabbage seed is imported to Nepal (32), and it is important to ensure that such seed is tested and free of the pathogen to minimise the risk of introduction of additional new races or pathotypes. Selection of cultivars with disease resistance is another means of control (12, 34, 43), which should be considered in combination with crop rotation and sanitation schemes.

V-shaped leaf lesions with black veins, typical of black rot (6, 44), were frequently observed in the fields, and *Xcc* was isolated from these lesions regardless of the crop growth stage. Blight symptoms, characterised by a sudden collapse of interveinal tissues and lack of veinal necrosis at earlier stages of infection, similar to those observed in Hawaii (29, 45) and in the UK (S.J. Roberts, unpublished), were not observed in the Nepalese fields. However, during race-typing under controlled conditions, we observed

both typical black rot symptoms and blight-like symptoms especially in plants inoculated with strains of races 5, 6 and 7. A previous study has also shown that inoculation of *B. oleracea* accessions with race 5 and 6 strains more commonly produced blight symptoms than when inoculated with other races (40). The blight symptoms appeared similar to those previously described (2) after artificial inoculation under greenhouse conditions.

In this study, duplicate isolates obtained from the same lesion produced similar rep-PCR profiles, except in one case where two isolates belonged to different races. This suggests that rep-PCR can be used for rapid initial screening of isolates to select non-identical ones for further analysis, including race-typing. The race affiliation of the two isolates from the same or adjacent lesions was tested twice to ensure that the result was reproducible. The results in general indicate that most leaf lesions, from which isolations were made, were a result of infection by a single race of the pathogen. However, the two races obtained from the same leaf indicate that mixed infections, or leaf lesions with a race mixture produced as the result of merged lesions from several infection points, may develop if more than one race is present in the field. This is not unlikely as different races were identified at the field level in more than half of the cabbage fields sampled in this study.

Rep-PCR of strains of *Xcc* from different geographic regions of Nepal produced fingerprinting profiles for races 1, 4 and 6 that clustered according to races and different lineages therein. Nepalese race 1 strains formed a single cluster, and strains of races 4, 5 and 6 were split into at least two clusters. All strains of race 4 and 6, except one strain of each race, clustered in two separate clusters, respectively. Previously, it has also been found that *X. campestris* pv. *raphani* and a selection of *Xcc* strains clustered in

accordance with races (40). In contrast, it was concluded on the basis of rep-PCR (with REP and BOX primers only) that fingerprints of *Xcc* strains from Tanzania (18) and Nepalese strains (27) were related to geographical origin, but the races of these strains were not determined and it is possible that different races were present in different areas. We found (data not shown) that the ERIC primers, which were not included in that study (18), are the most important for discriminating between *Xcc* races. ERIC-PCR also revealed a higher level of genetic diversity than REP and BOX primers in a previous study of *X. axonopodis* pv. *phaseoli* and pv. *phaseoli* var. *fuscans* (15). Another study showed that two *Xcc* race 3 strains had different rep-PCR fingerprinting profiles (or were grouped in different clusters) and suggested that race affiliation cannot be inferred by DNA fingerprinting (36). However, the 14 Israeli strains were not race-typed. More recently, results of fingerprints using BOX-PCR and M13-PCR of *Xcc* strains from Italy were not correlated with geographical and host origin (45). Other rep-PCR studies of other *Xanthomonas* species and pathovars have shown grouping of strains affiliated with distinct symptom groups (22), geographical origin (25) and year of isolations (26). This indicates that the potential to use rep-PCR to differentiate pathogenic variants within a pathovar may depend on several parameters.

Five races of *Xcc* were found in Nepal with races 1, 4 and 6 dominating. The dominance of races 1 and 4 has also been reported from other countries and the other races were considered rare (40). A previous study (39), also showed the presence and dominance of race 4, followed by race 1 and 6 amongst 51 strains from Portugal, mainly from *B. oleracea*; in that study race 4 accounted for 53% of the strains, race 6 for 29% and race 1 for 18%. In contrast, race-typing of 102 strains from the UK mainly from *B. oleracea*

(including 68 strains from cauliflower) showed that race 1 dominated (69%) followed by race 4 (30%); a single strain of race 3 was also identified in the UK collection (40). The presence of race 5 has previously been reported for only three strains of older origin (1953-1975) (40), which suggested that these races might not exist anymore. However, our study revealed that race 5 still exists, although at low frequency (7%). The two lineages of races 4 and 6 may suggest two different origins for these races, or that races 4 and 6 have evolved into two distinct lineages, which might even be different races, not yet identified by lack of discerning differentials.

Two strains, N35 and N47, were pathogenic and very aggressive on all genotypes except on *B. juncea* line FBLM2, and were assigned to race 7. Differences in the ability of the strains to cause disease in artificial inoculation tests were also revealed previously with Nepalese strains from the Katmandu and Chitwan valleys (1). Florida Broad Leaved Mustard (FBLM2) is compatible with (i.e. susceptible to) *Xcc* race 6, but not with the new race 7 (8, 13). It has been shown that mutants of Xcc race 1 with insertions or deletions in *avrXccFM* become virulent on Florida Broad Leaf Mustard (5). Similarly, a single gene difference may in this way determine the difference in compatibility between races 6 and 7 on Florida Broad Leaf Mustard. The rep-PCR clustering of the two races indicate a close relatedness between these two races, so it is possible that they may differ only in the presence of an *avr* gene.

More than one race was identified in all districts, and there were no obvious differences in the race composition within the geographically isolated regions sampled. Most cabbage seed is imported, and during sampling it became evident that one cultivar dominated in all growing areas. This may imply that the pathogen was introduced into the

areas with the seed. However, many factors play a role in the occurrence and diversity of the pathogen, for instance tight crop rotation schemes with cabbage and other Brassica crop species, and may together contribute to the occurrence and diversity of the pathogen.

Race-typing of strains is time, space and labour intensive. This study has shown that fingerprinting of strains using the combined rep-PCR profiles (REP, ERIC, and BOX primer sets) has potential to differentiate species, pathovars, pathotypes and lineages within *Xcc*. The presence of several races of the bacterium within Nepal, including the new race 7, emphasises the need for local surveys of *Xcc* to gain insight in the population composition and change. The results may be of value in relation to regulatory issues, such as seed import, but also highlight the need to account for pathogenic diversity when breeding, introducing and cultivating resistant cultivars to control the disease.

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Table 1. Cabbage growing areas in the lower hills of the Himalayas and the Kathmandu valley visited July 2001, the altitude, number of fields sampled with black rot symptoms, number of fields from which *Xanthomonas campestris* pv. *campestris (Xcc)* isolation was attempted, and the number of strains included in rep-PCR and race typing.

Region	Zone	District	Altitude (m)	Sampled fields with black rot symptoms	Fields from which isolation was carried out	Number of strains[a]	
						Pathogenicity test positive and rep-PCR	race-typed[b]
Western	Dhawalagiri	Baglung	1521-69	3	2	4 (+3)	4 (+1)
Central	Bagmati	Lalitpur	1430-84	7	5	13 (+7)	13 (+2)
Central	Bagmati	Bhaktapur	1296-1304	2	2	5 (+4)	5 (+1)
Central	Narayani	Makawanpur	1710-1950	7	3	6 (+6)	6 (+1)
Eastern	Koshi	Dhankuta	1734-2139	9	6	11 (+10)	11 (+2[c])
Total				28	18	39 (+30)	39 (+7 [c])

[a]Number of strains obtained from individual plants; parentheses indicate the number of duplicate strains that were also tested (+x).

[b]Race typing included one pair of strains from the same plant that had different rep-PCR profiles[c].

1

2

1

Table 2. Brassica accessions used in pathogenicity tests to identify *Xanthomonas campestris* pv. *campestris* races

Genotype	Species	Seed source[a]	Type	Interaction with race[b]						
				1	2	3	4	5	6	7
Wirosa F_1	*B. oleracea*	Bejo	Hybrid	+	+	+	+	+	+	+
Just Right Hybrid Turnip	*B. rapa*	Otis	Hybrid	+	+	+	-	+	+	+
Cob60	*B. napus*	WHRI	Doubled haploid	+	+	+	-	+	+	+
Seven Top Turnip	*B. rapa*	Otis	Open pollinated	+	-	+	-/+	-/+	+	+
Bo99075 (PI 199947)	*B. carinata*	WHRI	Inbred line	-	+	-	-	+	+	+
FBLM 2	*B. juncea*	Otis	Doubled haploid	-	+	-	-	-/(+)	+	-
Miracle F_1	*B. oleracea*	Bejo	Hybrid	+	-	-	+	-	(+)/+	+
SxD1	*B. oleracea*	WHRI	Doubled haploid	+	-	-	+	-	(+)/+	+

[a] Bejo= Bejo Zaden, Warmenhuizen, Holland; WHRI= Warwick HRI, The University of Warwick, UK; Otis=Otis S. Twilley Seed Co., US.

[b] +, compatible interaction (susceptible host); -, incompatible interaction (resistant host); (+), weakly pathogenic; -/+, variable result within accession. Adapted from Vicente *et al.* (2001) based on unpublished results and results of the present study.

Table 3. The origin of *Xanthomonas campestris* pv. *campestris* strains and the results of pathogenicity testing and race-typing.

HRI number	Original number [a]	Crop	District/ Field number	Pathogenicity Cph. Market [b]	Pathogenicity Wirosa	Race
Collected in 2001						
8429 A	N1	Cabbage	Lalitpur/2	+	nt	6[c]
8430	N5	Cabbage	Lalitpur/2	+	nt	4
8431 A	N6	Cabbage	Lalitpur/3	+	nt	4[c]
8432	N8	Cabbage	Lalitpur/3	+	nt	6
8434	N11	Cabbage	Lalitpur/4	+	nt	1
8435 A	N12	Cabbage	Lalitpur/4	+	nt	4
8388 A/B	N14/N15	Cabbage	Lalitpur/4	+	nt	4
8389	N16	Cabbage	Lalitpur/4	+	nt	4
8436 B	N18	Cabbage	Lalitpur/5	+	nt	4
8437 A	N25	Cabbage	Lalitpur/5	+	+	1
8390 A/B	N20/N21	Cabbage	Lalitpur/6	+	+	4[c]
8439 A	N22	Cabbage	Lalitpur/6	+	nt	1
8440	N27	Cabbage	Lalitpur/6	+	nt	4
8441 A	N29	Cabbage	Dhankuta/3	+	nt	1
8442 A	N31	Cabbage	Dhankuta/3	+	nt	1
8443 B	N34	Cabbage	Dhankuta/5	+	nt	5[c]
8444A	N35	Cabbage	Dhankuta/5	+	nt	7[c]
8445 A/B	N37/N38	Cabbage	Dhankuta/6	+	nt	1

8446	N40	Cabbage	Dhankuta/7	+	nt	6[c]
8447 A	N41	Cabbage	Dhankuta/7	+	nt	1
8448 A	N43	Cabbage	Dhankuta/8	+	nt	6[c]
8449 A	N45	Cabbage	Dhankuta/8	+	nt	4
8450 A	N47	Cabbage	Dhankuta/9	+	nt	7[c]
8450 B	N48	Cabbage	Dhankuta/9	+	nt	6[c]
8451 A	N49	Cabbage	Dhankuta/9	+	nt	6
8452 A/B	N51/N52	Cabbage	Makawanpur/1	+	nt	1
8453 B	N54	Cabbage	Makawanpur/1	+	nt	4
8454 A	N55	Cabbage	Makawanpur/4	+	nt	6[c]
8455 A	N57	Cabbage	Makawanpur/4	+	nt	1
8456 A	N59	Cabbage	Makawanpur/7	+	nt	1
8457 A	N61	Cabbage	Makawanpur/7	+	nt	1
8458 A/B	N63/N64	Cabbage	Baglung/1	+	nt	6[c]
8459 B	N66	Cabbage	Baglung/1	+	+	6
8460 B	N68	Cabbage	Baglung/2	+	nt	5[c]
8461 B	N70	Cabbage	Baglung/2	+	+	5
8462 A/B	N71/N72	Cabbage	Bhaktapur/1	+	+	1
8463 A	N77	Cabbage	Bhaktapur/1	nt	+	4
8464 B	N74	Cabbage	Bhaktapur/3	+	nt	4
8465 A	N75	Cabbage	Bhaktapur/3	+	nt	4
8466	N79	Cabbage	Bhaktapur/3	nt	+	4
Collected in 2003						
8467 A	N81	Cauliflower	Dhankuta/10	nt	+	4
8468 A	N83	Cauliflower	Lalitpur/7	nt	+	4
8469	N85	Cauliflower	Lalitpur/8	+	+	4

8471	N87	Leaf mustard	Lalitpur/10	+	+	6[c]
8472	N88	Cauliflower	Lalitpur/11	nt	+	4

[a] Two numbers indicate that duplicate strains from the same plant were tested with the same results.

[b] +, compatible interaction; nt, not tested.

[c] The duplicate strains or one strain were tested twice in two separate experiments.

Table 4. Frequency of occurrence of *Xanthomonas campestris* pv. *campestris* races among 40 strains obtained from cabbage plants in five districts in Nepal.

	Number of strains of race					
District	**1**	**4**	**5**	**6**	**7**	**Total**
Baglung			2	2		4
Lalitpur	3	8		2		13
Bhaktapur	1	4				5
Makawanpur	4	1		1		6
Dhankuta	4	1	1	4 [a]	2 [a]	12
Total	12	14	3	9	2	40
	(30.0%)	(35.0%)	(7.5%)	(22.5%)	(5%)	

[a] In one case, two races were obtained from the same lesion (plant).

Fig. 1. Cabbage growing areas (districts) from which leaves with typical black rot symptoms were collected in 2001 and confirmed to be caused by *Xanthomonas campestris* pv. *campestris* in the present study. (Map courtesy of Matt Rosenberg, Geography at About.com: http://geography.about.com).

Fig. 2. Dendrogram of the genetic similarity of 40 race-typed Nepalese *Xcc* strains from cabbage, four from cauliflower and one from leaf mustard, analysed together with two control strains of *Xcc* (HRI 1279A and 3811), and combined with previous fingerprinting data from 13 *Xcc* and eight other *Xanthomonas* sp. strains (including representative strains of *X. campestris* pvs. *raphani* and *incanae*, *X. campestris* strains from wallflower and candytuft, *X. vesicatoria* and *X. axonopodis* pv. *vesicatoria*) from Vicente *et al.* (2006). Race type strains of *Xcc* and *X. campestris* pv. *raphani* and type strain of *X. vesicatoria* are indicated with a T. Similarities were calculated from the combined data of PCR fingerprinting using repetitive extragenic palindromic (REP), enterobacterial repetitive intergenic consensus (REP) and BOX primer sets. Strain numbers without a prefix are HRI numbers. Solid and dashed lines delimit the most important groups of strains at 70 and 80% similarity, respectively.

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