Discrimination of *Xanthomonas campestris* pv. *campestris* races among strains from
northwestern Spain by Brassica spp. genotypes and rep-PCR

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Abstract

Black rot, caused by *Xanthomonas campestris* pv. *campestris* (Xcc), is a severe seedborne disease of Brassica crops around the world. Nine races are recognized, being races 1 and 4 the most aggressive and widespread. The identification of Xcc races affecting Brassica crops in a target area is necessary to establish the adequate control measures and breeding strategies. The objectives of this study were to isolate and identify Xcc strains from northwestern Spain Brassica crops, by using semi-selective medium and pathogenicity tests, determine the existing races of Xcc in this area by differential series of *Brassica* spp., and evaluate the use of repetitive DNA polymerase chain reaction-based fingerprinting (rep-PCR) to differentiate among the nine existing Xcc races. Seventy five isolates recovered from infected fields were identified as Xcc. Race-typing tests determined the presence of the following seven pathogen races: 1, 4, 5, 6, 7, 8 and 9. Race 4 was the most frequent in *Brassica oleracea* and race 6 in *Brassica rapa* crops, therefore breeding should be focussed in obtaining resistant varieties to both races. Cluster analysis derived from the combined fingerprints showed four groups, but no clear relationship to race, crop or geographical origin was found. Rep-PCR analysis was found not to be a reliable method to discriminate among Xcc races, therefore race typing of new Xcc isolates from other fields should be done by using the differential series of *Brassica* spp. genotypes or another alternative approach.

Introduction

Black rot, caused by *Xanthomonas campestris* pv. *campestris* (Xcc), is a destructive and seed-borne disease affecting Brassica crops worldwide. The disease becomes a constraint especially on vegetable Brassica production, causing lesions on leaves that reduce the commercial value of the product on the fresh market. The successful spread of the pathogen around the world can be associated with the frequent long-distance trade of contaminated seeds. In fact, seed companies and distributors are forced to spend time and resources checking the sanitary state of imported and exported seed lots.

Production and consumption of Brassica crops in Spain is quite relevant in the international market, especially regarding the import of cabbage and the export of cauliflower and broccoli. Black rot limits the vegetable Brassica production in Spain, particularly in warm and humid conditions (i.e. greenhouse production). Typical symptoms of the disease as chlorotic lesions and blackened veins on Brassica leaves were firstly described in Spain by Urquijo et al. (1971). This finding was later confirmed by Ortega and López (1990) and Lema et al. (2008), who identified and characterized Xcc strains from crops in the Mediterranean coast and northwestern Spain. In this last region, Brassica cultivation is basically made by numerous growers who trade their products in local markets. The small size of the field plots in this area and the seldom rare use of healthy plant material or disease-free seed could have helped contributed to the efficient dissemination of the pathogen. Recently, screenings for resistance in this area have been conducted (Lema et al. 2011), but no studies involving the pathogen have been accomplished until now.

Management of black rot is difficult and is usually attempted through the use of disease free planting material (seeds or transplants) and the elimination of other potential inoculum sources such as infected crop debris and cruciferous weeds (Taylor et al. 2002). The existence of pathogen races hampers crop breeding for black rot resistance as it requires a thorough knowledge on the prevalent ones in the growing area, to assist the plant breeder in obtaining appropriate resistant cultivars. Based on the interaction between Xcc and four differential host genotypes, Kamoun et al. (1992) have defined for first time five Xcc races defined by Kamoun et al. (1992) which were later confirmed by Ignatov et al. (1998). Afterwards, Vicente et al. (2001) modified the previous classification, deleting the previously described race 3, adding a new race and recategorizing the remaining races. They also established the current differential *Brassica* spp. series currently used for race determination, suggested a set of reference strains for the six races and proposed a gene-for-gene model to explain the interactions between the races and the differential accessions. Recently, Fargier and Manceau (2007) added three more races (7, 8 and 9) and confirmed the presence of a new resistance gene (R5) present in *B. juncea* 'Florida Broad Leaf Mustard' conferring resistance to race 7 (Avr 5). The model presented was the simplest hypothesis involving the smallest number of genes necessary to explain the observed interactions. Gene homology was assumed for cultivars with the same reaction pattern (Fargier and Manceau 2007).

Over the last years, several surveys were carried out aiming at identifying the existing Xcc races in diverse Brassica cultivation areas in Germany (Griesbach et al. 2003), Portugal (Vicente 2004) and Nepal (Jensen et al. 2010). It is widely documented that races 1 and 4 are the most virulent and widespread, accounting for most black rot cases around the world (Ignatov et al. 1998; Vicente et al. 2001; Griesbach et al. 2003;

Vicente 2004; Griffiths and Roe 2005; Jensen et al. 2010) even among cruciferous weeds (Ignatov et al. 2007). Strains of races 2, 3 and 5 are uncommon and race 6 has been reported only in *B. rapa* (Vicente 2004).

The method to discriminate among races based on the reactions of a set of differential *Brassica* spp. is time consuming as it requires growing the host genotypes, inoculating them and waiting until disease symptoms appear. Repetitive DNA polymerase chain reaction-based fingerprinting (rep-PCR) is a rapid, low-cost, and reliable diagnostic method that has already been extensively used to assess the genetic diversity and/or to study the phylogeny and taxonomy of *Xanthomonas* species affecting different crops (Lopes et al. 2001; Massomo et al. 2003; Mkandawire et al. 2004; Lopez et al. 2006; Mahuku et al. 2006; Jensen et al. 2010) and wild cruciferous plants (Ignatov et al. 2007). Vicente et al. (2006) and Jensen et al. (2010) found that the same Xcc races tend to cluster together based in rep-PCR fingerprinting data. Thus, a thorough evaluation of this method should be done to determine if it could become a reliable alternative to the use of differential series of *Brassica* spp, in discriminating Xcc races.

The objectives of this study were to (i) isolate and identify *Xanthomonas campestris* pv. *campestris* strains from northwestern Spain Brassica crops by using semi-selective medium and by pathogenicity tests (ii) determine the existing races of Xcc by using differential cruciferous genotypes, and (iii) evaluate the use of repetitive DNA polymerase chain reaction-based rep-PCR to differentiate among the nine existing Xcc races.

Materials and methods

Collection, isolation and identification of the pathogen

Xcc strains were recovered from symptomatic plants of different crops from *B. oleracea* and *B. rapa* plants species during surveys conducted from July to October in 2009 and 2010, in different fields located in northwestern Spain (Table 1). Leaves showing symptoms like those typical of caused by Xcc infections were collected and dried among paper sheets at room temperature before isolation of the causal agent. In most cases, one a leaf with characteristic black rot symptoms (typical leaf edge damages, with large Vshaped chlorotic lesions presenting necrotic areas with blackened veins) was collected from 5 individual plants in each field. From each selected leaf, tissues samples (0.25 cm²) were excised from the lesion margin, placed for 30 seconds in a 20% chlorine solution, washed 7-8 times in sterile distilled water, and cut and crushed mixed with a razor blade with 8-10 drops of sterile distilled water on a Petri dish. Loops of the resulting macerate were streaked onto Fieldhouse-Sasser (FS) starch-containing semi-selective medium (Schaad et al. 2001), which has been proved to be efficient in recovering Xcc from infected samples (Fukui et al. 1994; Koenraad et al. 2005). The plates were incubated at 32°C during 2-3 days and, after that, three yellow, mucoid colonies presenting zones of starch hydrolysis, indicating the presence of Xcc colonies, were selected out of those recovered from each leaf sample, and subcultured on the bacteria screening 523 medium (Sigma-Aldrich Chemie GmbH, Steinheim, Germany).

Pathogenicity tests of all collected strains were carried out on susceptible Savoy cabbage cv. Wirosa F1. Seventy five Xcc strains isolated from Spanish northwestern fields were used in this work (Table 1). Fifty eight strains were isolated from *B.*

oleracea crops, and 17 strains from *B. rapa*. Eighteen strains were duplicates obtained from the same lesion of the infected plants (12 duplicate Xcc strains from *B. oleracea* and 6 from *B. rapa*) (Table 1). Five additional Xcc strains, collected previously in from other areas from center and east of Spain and kindly supplied by M.M. López (Centro de Protección Vegetal y Biotecnología, IVIA, Valencia, Spain), were also included in this study. All these strains, totalling 80, were analyzed for race typing and rep-PCR. The All strains were maintained at -80° C in 2 ml eppendorf tubes containing 0.5 ml of 80% glycerol and 1ml of Xcc culture in yeast extract tryptone (YT) medium (prepared with 8 g l⁻¹ of tryptone, 5 g l⁻¹ of yeast extract, 2.5 g l⁻¹ of NaCl, pH 7.0) after shaking for 12-15 h at room temperature.

Four seeds of Savoy cabbage cv. Wirosa F1 were planted in 4 l pots with 19 cm diameter. To prepare the inoculum, Xcc strain cultures were streaked onto plates of screening 523 medium and grown for 48 h at 32°C. Cells were suspended in sterile distilled water and adjusted to an optical density of 0.50, at 600 nm, which corresponds approximately to $ca\ 5 \times 10^8$ cfu ml⁻¹. The multiple needle method described by Lema-Marquez et al. (2007) was used to inoculate the youngest leaf of each plant, (4 to 5 weeks old after planting). After inoculation, greenhouse conditions were set to 14 h of light, mean temperature 28/24°C (day/night) and relative humidity above 90% to favour development of black rot. Symptoms were recorded at 7 and 10 days post after inoculation. If no visible symptoms were detected then the interaction was considered as incompatible (-). If the leaf showed symptoms ranging from chlorotic lesions around the inoculation point to large V-shaped lesions with blackened veins then the interaction was considered as compatible (+). Strains which showed that caused weak or non pathogenic reaction were reinoculated.

Race typing

A differential *Brassica* spp. series, including six cultivars and lines belonging to five *Brassica* species, described by Vicente et al. (2001), was used for race typing (Table 2). The differential series included cv. 'Wirosa F1' (Savoy cabbage, *B. oleracea* supplied by Germisem, Spain), doubled-haploid line S×D1 (*B. oleracea*), doubled-haploid line Cob60 (*B. napus*, derived from line 14R of cv. Cobra), cv. 'Seven Top Turnip' (*B. rapa*), inbred line PIC 1 (*B. carinata*, selected from PI 199947), and doubled haploid line Florida Broad Leaf Mustard2 (Indian mustard, *B. juncea*, derived from 'Florida Broad Leaf Mustard') supplied by Warwick-HRI, Wellesbourne, UK. Four plants of each differential genotype were inoculated. The methodology concerning inoculum preparation, inoculation method, incubation conditions and rating of the symptoms were the same as for the pathogenicity test. Strains were classified into race types depending on their reaction in the differential *Brassica* spp. series (Table 2) following the model proposed by Vicente (2001) and Fargier and Manceau (2007).

Rep-PCR analysis

Eighty Spanish strains (Table 1) and the nine reference or type strains (Table 3) were used for rep-PCR analysis. Type strains for races 1 to 6 were kindly supplied by Warwick HRI (Wellesbourne, UK) and type strains for races 7, 8 and 9 were provided by CFBP-INRA (Angers, France). For DNA extraction, cultures from single colonies were grown in the 523 medium as mentioned in the previous section. Modified CTAB procedure described by Kieser et al. (2000) was used for bacterial DNA extraction.

DNA was quantified using a spectrophotometer (Spectra MR, Dynex Technologies) and final DNA concentrations were adjusted to 50 ng/μl and stored at -20 °C before use.

For each rep-PCR reaction a 10 μl reaction mixture was prepared with the following Reagents as follows: 1x Gitschier buffer, 10% (v/v) DMSO, 5 mM of dNTP mix, 2 μM of each primer, 5 ng/μl of DNA, 0.75 U of Taq DNA polymerase (BioTaq, Kapa Biosystems) and sterile MilliQ water up to 10 μl.. Primers used in this study were REP-PCR primers 1R (5'-III ICG ICGICA TCI GGC-3') and 2I (5'-ICG ICT TAT CIG GCC TAC-3'), the ERIC-PCR primers 1R (5'-ATG TAA GCT CCT GGG GAT TCA C-3') and 2I (5'- AAG TAA GTG ACT GGG GTG AGC G-3') and the BOX-PCR primer 1R (5'-CTA CGG CAA GGC GAC GCT GAC G-3') (Louws et al. 1994). Amplification reactions were performed using a PTC-100 TM Thermal Cycler (MJ Research, Watertown, MA, USA) following the method described by Rademaker and de Bruijn (1997) and Jensen et al. (2010). PCR products were examined by electrophoresis in 1.2% agarose 15-lane gels in 1 x TBE buffer solution for 4 h at 120 V. A molecular mass marker (0.07-19.3 Kbp DNA Ladder, Roche) was loaded on the left lane of each gel. Gels were stained in ethidium bromide and DNA visualized under UV light. Strains were tested twice by each rep-PCR method under each set of conditions, to ensure reproducibility of the DNA fingerprints.

For the analysis of rep-PCR fingerprints GelCompar II software package (Applied Maths, Kortrijk, Belgium) was used. Fingerprints were normalized according to intragel size standards. Bands sized between 925 and 3,500 bp were used. Data from BOX, ERIC and REP fingerprintings were combined and similarity matrices of genomic fingerprints were calculated using the Pearson product-moment correlation coefficient.

Cluster analyses were performed using the unweighted pair group method with arithmetic averages (UPGMA). A preliminary cluster analysis was carried out and several groups were defined. Parameters of band comparison (curve smoothing and tolerance) were optimized in order to maximize differences among already defined groups and a new cluster analysis was then performed with optimized parameters. Cophenetic values were computed to test the reliability of internal branches in the cluster analysis. Also the cophenetic correlation coefficient was calculated to test for the goodness-of-fit between similarity matrix obtained from the clustering dendrogram and the original similarity matrix using the MxCOMP procedure of NTSYS statistical package (Castaneda et al. 2005).

Results

Collection, isolation and identification of the pathogen

Strains were isolated from 19 fields in 15 locations representing different geographical areas (Table 1). In several plants, examination of leaves apparently showing black rot V-shaped typical lesions did not reveal the presence of Xcc. *B. rapa* was surveyed only in 3 of the 19 fields. In northwestern Spain, turnip greens are planted mainly in September and collected from January to March, when weather is not so favorable for the disease development.

A total of 75 strains showing yellow, mucoid colonies presenting zones of starch hydrolysis in semi-selective medium, were recognized as Xcc. All strains, but MBG-70.2 and MBG-76.1, showed interaction when inoculated onto cv. Wirosa F1. From the

five strains supplied by Centro de Protección Vegetal y Biotecnología, two were weakly pathogenic (MBG-151.1 and MBG-152.1) on that indicator plant, therefore races could not be clearly identified.

Race typing

All races were found among northwestern Spain Xcc strains, except races 2 and 3. Race 4 was the most common, but it only appeared in *B. oleracea* crops (Table 1). Contrarily, race 6 was the most common in turnip greens but it was not detected in any *B. oleracea* crop examined. In this crop, 45 strains were identified as race 4, five strains as race 9, three strains as race 1, two strains as race 5 and one strain as race 7. Regarding *B. oleracea*, cabbage strains belonged to races 1, 4, and 9, race 4 being the most common while kale strains belonged to races 4, 5, 7 and 9, with race 4 also being the most common. In *B. rapa*, 12 strains were identified as race 6, two strains as race 5, two strains as race 8 and one strain as race 7. Of the pathogenic isolates obtained from Centro de Protección Vegetal y Biotecnología, three were assigned to race 1.

Duplicate isolates represent strains recovered from the same lesion. In most cases, these duplicates were found to belong to the same race, except in the four following cases: MBG-36.1 (race 5) and MBG-36.3 (race 7); MBG-39.2 (race 6) and MBG-39.3 (race 7); MBG-70.1 (race 4) and MBG-70.2 (weakly pathogenic); and MBG-141.1 (race 5) and MBG-141.2 (race 8).

Rep-PCR analysis

PCR amplifications generated distinctive gel band patterns among the Xcc strains when tested with each of the three set of primers used. Nevertheless, the number of distinctive bands amplified with each primer set was restricted: 8 bands ranging between 1,208 and 3,121 bp with REP, 5 bands between 1,124 and 2,258 bp with ERIC and 10 bands between 1,006 and 3,154 bp with BOX. A dendrogram was created by combining the 23 distinctive bands generated by ERIC, REP and BOX primers in rep-PCR (Figure 1).

A cophenetic correlation coefficient value of 0.86 was achieved, indicating a good fit between similarity matrix obtained from the cluster dendrogram and the original similarity matrix. The reliability of internal branches of the dendrogram was also measured by cophenetic values (Figure 1). Four clusters are identified at 60% similarity. The Cluster 1 contains 8 strains from northwestern Spain belonging to race 4 (six strains obtained from kale and two strains from cabbage). The Cluster 2 consists of 50 strains obtained from different geographical origins and crop, including all reference strains except race 8. In this cluster strains from northwestern Spain belong mainly to race 4 (30 strains) although races 1 (three strains), 6 (two strains), 7 (one strain) and 9 (three strains) are also represented. In this group three strains that are weakly pathogenic on Wiroso F1 are also included. The Cluster 3 includes 15 strains, of which 10 are from several locations at northwestern Spain, 4 are from east and centre of Spain (one was a weak-pathogenic strain) and 1 is the type-strain of race 8 (from France). The Cluster 4 contains almost all strains collected from turnip greens, which belong mostly to race 6, although races 5 (two strains from kale and two from turnip greens), 7 (one strain from kale) and 8 (two strains from turnip greens) are also represented.

The reference strains, except race 8, are grouped together into the cluster 2. These reference strains are from very diverse geographical origins (Table 3). Reference strains for races 3, 6 and 9 group together at 84% similarity and close to race 2 (80% similarity). Furthermore races 1 and 7 show similar band profiles (91% similarity). The Race 8 reference strain is placed in cluster 3.

No clear relationship is found between the groups defined in the cluster analysis and the race affiliation of the strains. However, race 4 strains are mainly distributed in clusters 1 and 2, race 1 strains are placed are in clusters 2 and 3, and race 5 and 8 strains are placed in cluster 4. Groups defined in the cluster analysis do not reveal a clear relationship with their geographical origin and/or their crop host. However, cluster 1 is composed by race 4 strains, mainly isolated from kale, and cluster 4 is entirely composed by strains isolated from close geographical locations mostly from turnip greens. On the other hand, some strains show similar band profiles, but they belong to different races (Figure 1). For example, strains 145.1 and 145.3 present a 95% similarity and they belong to races 5 and 8, respectively. Similarly, strains MGB-38.3 and MGB-39.3 with a 97% similarity belong to race 6 and 7, respectively.

Discussion

Although black rot disease is widely distributed in northwestern Spain, little is known about the causal agent, Xcc, presence or race diversity. Until now, no exhaustive studies on an collection, isolation, identification, and race-typing of Spanish Xcc strains had been accomplished. However, other authors have included a few Xcc strains originated from Spain in their works as Vicente et al. (2001), who included one strain from Spain

belonging to race 4, in their study concerning the occurrence and geographical distribution of Xcc races. Moreover Fargier and Manceau (2007) in their studies to clarify the race composition of Xcc populations, included four Spanish strains isolated from cauliflower, three of which were race-typed as race 1 and one as race 9.

In the present work and for the first time, Xcc strains from one of the most important Brassica consumer and producer country in Europe are studied in detail. Morphological characteristics assigned xanthomonad-like bacteria to the species as the development of starch hydrolysis zones around colonies on semi-selective medium and the results of pathogenicity tests provided the definitive evidence that strains included in this study were *X. campestris*. Nevertheless, several symptomatic leaves showing apparently black rot lesions did not reveal the presence of Xcc. This is not surprising as symptoms similar to those caused by Xcc may be due to plant senescence or caused by the fungus *Leptosphaeria maculans* as pointed out by Williams (1980) and Jensen et al. (2010), respectively. According to Williams (1980), the initial stages of black leg, caused by the fungus *Leptosphaeria maculans*, can be easily confounded with black rot symptoms in Brassica crops. Also senescence process can be confused with black rot symptoms (Jensen et al. 2010). Of the Xcc populations under study, only four strains were weakly pathogenic on Savoy cabbage cv. Wirosa F1 thus hampering race identification (Table 1). This kind of reaction was also found by Vicente et al. (2001), who attributed the loss of pathogenicity of some strains to poor maintenance conditions or inappropriate long-term preservation under laboratory conditions. Nevertheless, Griesbach et al. (2003) assumed that levels grades of virulence can occur in different Xcc strains independently of geographic origin or race. Currently, nine races are proposed for *X. campestris* pv. *campestris* based on the response of certain Brassica species and cultivars (Vicente et al.

2001; Fargier and Manceau 2007) with races 1 and 4 being predominant (Vicente et al. 2001). In our work, most races were found among strains recovered from northwestern Spain crops, except races 2 and 3, which are considered uncommon (Vicente 2004). On the other hand race 5, which is also considered uncommon (Vicente 2004), was found in a Xcc population recovered from an infected field in Salcedo (Table 1). Considering that only a small region was surveyed, our data shows that there is high race variability among the strains collected from northwestern Spain. This contrasts with results obtained by Griesbach et al. (2003) who only found races 1 and 4 after when race-typing strains from Italy and Germany, and by Vicente (2004) who race-typed 51 strains from Portugal and found 18% belonging to race 1, 53% to race 4 and 29% to race 6; however it should be noted that races 7 to 9 were not defined at the time. Fargier and Manceau (2007) found races from 1 to 9 after analyzing 32 Xcc strains from all over the world and Jensen et al. (2010) working with races recovered from Nepal, found races 1, 4, 5, 6 and 7, races 1, 4 and 6 being the most frequent.

The high race variability among northwestern Spanish Xcc strains may be due to multiple introductions of the black rot pathogen into Spain, probably associated with the frequent entry of contaminated seed. High race variability was also found in the fields of the experimental station (MBG-CSIC) at Salcedo namely fields 11, 12 and 13 (Table 1). This may be due to the nearby explained because a Brassica Germplasm Bank is maintained at Salcedo which contains more than 525 local accessions and its seed stock has been collected directly from the farmer fields since the 1980's. In our agricultural system, landraces regenerated by farmers from year to year are mostly used. The use of commercial disease-free seed is uncommon, thus maximum variability of this seed-borne pathogen can be expected in those materials.

In this work, race 4 was found to be predominant in *B. oleracea* crops, whereas race 6 was found only in *B. rapa*. Ignatov et al. (2007) found a high frequency of races 5 and 6 on weedy crucifer populations in California. They suggested that races of Xcc were derived originally from the abovementioned two races by mutation and accumulation or modification in avirulence genes. The authors also state that races 5 and 6 are rare on *B. oleracea* crops although they are able to can infect them after inoculation. This is supported by our results where all strains belonging to race 6 were found in *B. rapa*.

Also turnip greens are traditionally grown in northwestern Spain, where no commercial seed is extensively imported. This fact can determine that the race occurrence and diversity of Xcc in *B. rapa* be more restricted than that in major *B. oleracea* crops like cabbage or cauliflower, where genetic diversity can be mostly influenced by the introduction of the pathogen by means of infected commercial seeds or seedlings. Other works showed race 1 to be predominant in *B. oleracea* crops, followed by race 4 (Vicente et al. 2001; Fargier and Manceau 2007), whereas our study showed race 4 to be predominant in those *B. oleracea* crops widely grown in northwestern Spain. Race 1 was found only among strains isolated from cabbage crops in one field in Vilanova de Arouca and in three isolates supplied by Centro de Protección Vegetal y Biotecnología and originated recovered from commercial varieties of cabbage and cauliflower. Probably race 4 is an endemic race of northwestern Spain infecting kale varieties. These are highly variable landraces that may have coexisted with race 4 for years until the arrival of commercial varieties which could have brought the carrying race 1 into this area.

The results here obtained extend our knowledge on the genetic diversity of Xcc and are very useful regarding the black rot disease management based on host resistance. As a conclusion and because of race frequency, breeding programmes designed to provide resistance, at least to race 4 in *B. oleracea* and race 6 in *B. rapa*, must be developed so that new released plant materials with improved resistance to Xcc are available to growers in northwestern Spain.

Louws et al. (1999) demonstrated that rep-PCR genomic fingerprinting is a useful tool for molecular characterization and for genetic diversity assessment of different bacterial genera including *Xanthomonas*. Since then, rep-PCR has been widely used for identification and classification of *Xanthomonas* and other bacterial genera at species, sub-species (Lema-Marquez et al. 2007), pathovar (Louws et al. 1995; Barak & Gilbertson 2003; Jensen et al. 2010) and sub-pathovar levels (Louws et al. 1995; Lopez et al. 2006). Valverde et al. (2007) confirmed that rep-PCR shows a potential to determine the diversity within Xcc similar to that of other techniques like pulsed field gel electrophoresis (PFGE) or amplified fragment length polymorphism (AFLP). Diverse studies exploring the diversity in *X. campestris* revealed that this species comprises genetically heterogeneous (i.e. *vesicatoria*, and *campestris*) and homogeneous (i.e. *pelarogonii*, *vitians*, *musacearum*) pathovars. However, according to Louws et al. (1999) and Barak and Gilbertson (2003) rep-PCR can only prove intraspecific variability among strains of heterogeneous pathovars. Thus, the finding that pathogenic Xcc strains analysed in our study showed different rep-PCR fingerprints was expected, being consistent with previous studies and further confirms that *campestris* is a heterogeneous pathovar.

After performing rep-PCR fingerprinting, a total of 23 distinctive bands were found among the analyzed strains. Valverde et al. (2007) found 29-41 bands ranging in size between 200 and 3,000 bp based on BOX, ERIC and REP profiles of 22 different Xcc strains, mainly obtained from Israel, whereas Tsyngakova et al. (2004) using rep-PCR to study the genetic relationships among 51 *X. campestris* strains collected from different countries worldwide found 60 bands. Although the number of distinctive bands found in our work is lower than that reported in abovementioned studies, we have to take into account that all of the above authors worked with strains representing other *Xanthomonas* pathovars, and this fact could have maximized the distances among groups. In the present study, only a smaller number of bands were detected with ERIC primers compared to those detected with BOX or REP. On the contrary, Jensen et al. (2010) stated that ERIC primers were the most important to discriminate among Xcc races. The use of ERIC primers also revealed a higher level of genetic diversity than that revealed when using REP and BOX primers in *X. campestris axonopodis* pv. *phaseoli* and contributed to the identification of two clusters associated with the strain geographical origin (Lopez et al. 2006).

In our work, rep-PCR analysis did not cluster strains based on their geographical origin. Similar results were found by Jensen et al. (2010), Valverde et al. (2007) and Zaccardelli et al. (2008), who studied Xcc strains from Nepal, Israel and Italy, respectively. On the other hand, Massomo et al. (2003) showed that specific rep-PCR genomic fingerprints of Xcc strains were linked to some geographical areas in Tanzania. Zhai et al. (2010) reported a possible relationship between virulence and/or geoclimatic origin of the worldwide strains of another pathovar of the same species named *X. campestris* pv. *malvacearum*.

In the present study, rep-PCR did not show adequate genetic differentiation to clearly discriminate among the nine Xcc races although Vicente et al. (2006) and Jensen et al. (2010), had found a tendency for the same races to cluster together based on rep-fingerprinting, Jensen et al. (2010) found that race 4 strains were divided between two distinct clusters and that strains from races 6 and 7 clustered together. Xcc races are differentiated by a small number of resistance genes in the plant, which corresponded with to the same number of avirulence genes in the pathogen (Table 2). Therefore, it was not surprising that those avirulence genes and thus the race affiliation could be detected by the analysis of repetitive DNA sequences. Based on previous studies, rep-PCR appeared to be an adequate typing method for *X. campestris* identification at pathovar or sub-pathovar level. However, our results proved otherwise, as they clearly show that rep-PCR was not suitable for Xcc race differentiation. This must be accomplished by pathogenicity tests carried out on differential sets of *Brassica* spp. coupled with *in vitro* properties such as growth in semi selective medium, or by developing markers linked to the avirulence genes that determine the race affiliation of each strain.

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bacterial races 7, 8 and 9. Authors also thank to Eva González her help in DNA extractions.

Figure legends

Figure 1. Dendrogram of the genetic similarity of 80 *Xanthomonas campestris* pv. *campestris* strains from Spain and nine reference strains based on 23 polymorphic bands. The scale at the top indicates the degree of genetic relatedness between strains. Numbers at the nodes of clusters represent the cophenetic correlation values. The names of the polymorphic markers found for the set of strains analyzed are shown at the top of the graphic. Strains with identical symbol belong to the same cluster.

Table 1. Geographical origin, crop and race assignation of strains of *Xanthomonas campestris* pv. *campestris* recovered in northwestern Spain utilized in this study.

Province	Location	Field	Crop	Race [†]	Strain [‡]
A Coruña	Oroso	1	Cabbage	4	MBG-89.1, MBG-90.1, MBG-90.2
			Kale	4	MBG-96.1
				9	MBG-97.1, MBG-101.1
	Santiago de Compostela	2	Kale	4	MBG-167.1, MBG-168.3
	Trasmontes	3	Kale	4	MBG-82.1, MBG-83.1
Ourense	Leiro	4	Kale	4	MBG-107.1
	Melón	5	Kale	4	MBG-119.1
	Barro	6	Kale	4	MBG-160.1
Pontevedra	Lalín	7	Cabbage	4	MBG-135.1, MBG-135.2, MBG-136.1, MBG-136.2, MBG-137.1, MBG-137.2, MBG-138.1
			Kale	4	MBG-139.1, MBG-139.2, MBG-140.1, MBG-140.2, MBG-158.1
		8	Cabbage	4	MBG-74.1, MBG-74.2
			Kale	9	MBG-78.1
				WP	MBG-76.1
	Maúnzo	9	Cabbage	4	MBG-45.1
			Kale	4	MBG-68.1, MBG-70.1, MBG-72.1
				WP	MBG-70.2
	Ribadumia	10	Cabbage	9	MBG-164.1

		Kale	9	MBG-161.1
Salcedo	11	Turnip greens	6	MBG-38.3, MBG-39.2, MBG-42.1
			7	MBG-39.3
			5	MBG-29.1
	12	Turnip greens	6	MBG-23.1, MBG-24.1, MBG-26.1, MBG-28.1, MBG-29.2, MBG-30.1, MBG-30.2, MBG-30.3, MBG-33.1
			5	MBG-36.1, MBG-36.2
			7	MBG-36.3
	13	Turnip greens	5	MBG-145.1
			8	MBG-145.2, MBG-145.3
	14	Kale	4	MBG-103.1, MBG-104.1, MBG-104.2, MBG-105.1
	Sobral	15	Cabbage	4
Kale			4	MBG-46.1, MBG-57.1, MBG-58.1, MBG-58.2, MBG-60.1
16		Cabbage	4	MBG-47.1
Soutelo de Montes	17	Kale	4	MBG-156.1
Vigo	18	Kale	4	MBG-142.1, MBG-143.1
Vilanova de Arousa	19	Cabbage	1	MBG-44.1, MBG-61.1, MBG-63.1
		Kale	4	MBG-43.1, MBG-67.1
Castellón	Almenara	Cauliflower	WP	MBG-152.1
	Benicarló	Kale	WP	MBG-151.1
Madrid	Madrid	Cabbage	1	MBG-154.1

Menorca	Es Castell	Cauliflower	1	MBG-153.1
Valencia	Cuatrecorba	Cauliflower	1	MBG-150.1

[†]WP: Weakly pathogenic

[†]Duplicates are designated with the same number before the dot

Table 2. Reaction of the differential Brassica spp.series to the nine races of <i>Xanthomonas campestris</i> pv. <i>campestris</i> , after Vicente et al. (2001) and Fargier and Manceau (2007) differential series	Resistance (R) genes	Races /Avirulence genes								
		1	2	3	4	5	6	7	8	9
		A1	A2, A3	A1, A3	A1?, A4	A3, A5	-	A5	A1, A2, A3	A1, A3, A4
Cob60 (<i>B. napus</i>)	R4	+	+	+	-	+	+	+	+	-
FBLM2 (<i>B. juncea</i>)	R1, R4?, R5	-	+	-	-	(+)	+	-	-	-
PIC 1 (<i>B. carinata</i>)	R1, R4?	-	+	-	-(+)	+	+	+	-	-
Seven Top Turnip (<i>B. rapa</i>)	R2, R4	+	-	+	-	+	+	+	-	-
SxD ₁ (<i>B. oleracea</i>)	R3	+	-	-	+	-	+	+	-	-
Wirosa F ₁ (<i>B. oleracea</i>)	--	+	+	+	+	+	+	+	+	+

+ = compatible interaction, - = incompatible interaction, (+) = partially incompatible interaction.

Table 3. Reference strains for the nine races of *Xanthomonas campestris* pv. *campestris*.

Identification	Race	Host	Origin
HRI 3811	1	<i>Brassica oleracea</i>	USA
HRI 3849A	2	<i>Brassica oleracea botrytis</i>	USA
HRI 5212	3	<i>Brassica oleracea botrytis</i>	United Kingdom
HRI 1279A	4	<i>Brassica oleracea capitata</i>	United Kingdom
HRI 3880	5	<i>Brassica oleracea capitata</i>	Australia
HRI 6181	6	<i>Brassica rapa</i>	Portugal
CFBP 4953	7	<i>Brassica oleracea botrytis</i> cv. Cortes	Belgium
CFBP 1124	8	<i>Brassica oleracea botrytis</i>	France
CFBP 6650	9	<i>Brassica oleracea</i>	United Kingdom