Project title:	Characterisation of *Xanthomonas campestris* pv. *campestris* isolates from the 2019 outbreak of black rot of crucifers in Cornwall
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Key staff:	Joana G. Vicente (Visiting Research Fellow) Josie Brough (Research Technician) Professor Eric B. Holub (Group Leader) School of Life Sciences, Wellesbourne Campus, University of Warwick, Warwick CV35 9EF
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Industry Representative:	N/A
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The results and conclusions in this report are based on an investigation conducted over a five month period. The conditions under which the experiments were carried out and the results have been reported in detail and with accuracy. However, because of the biological nature of the work it must be borne in mind that different circumstances and conditions could produce different results. Therefore, care must be taken with interpretation of the results, especially if they are used as the basis for commercial product recommendations.

AUTHENTICATION

We declare that this work was done under our supervision according to the procedures described herein and that the report represents a true and accurate record of the results obtained.

[Name]

[Position]

[Organisation]

Signature ..... Date .....

[Name]

[Position]

[Organisation]

Signature ..... Date .....

Report authorised by:

[Name]

[Position]

[Organisation]

Signature ..... Date .....

[Name]

[Position]

[Organisation]

Signature ..... Date .....

CONTENTS

GROWER SUMMARY

Headlines

- Ten isolates obtained from samples of cauliflower from Cornwall caused typical black rot symptoms in a range of brassicas and were shown to belong to two different races, 1 and 4, with race 4 being predominant
- Molecular testing of the isolates confirmed that they are *Xanthomonas campestris*
- PCRs with pathovar-specific primers were inadequate to differentiate *X. campestris* pvs. *campestris*, *raphani* and *incanae* whilst PCRs with primers specific for races 1 and 4 show potential for identification of race 1, but not race 4

Background

Black rot of crucifers is one of the most important diseases of brassicas worldwide and it occurs regularly in UK vegetable brassica crops. The disease is favoured by warm and humid conditions and tends to vary in importance from year to year possibly due to different levels of contamination of seeds and transplants and variable weather conditions.

Control of this disease is complicated as there are no chemicals approved and generally it relies mostly on testing of seeds and good hygiene practices in plant nurseries and fields.

Black rot of crucifers is caused by the bacterium *Xanthomonas campestris* pv. *campestris*. All brassicas can be infected including vegetable brassicas from different species (e.g. *Brassica oleracea* like cauliflower and cabbage*, B. rapa* like turnips and Chinese cabbage, *B. napus* like swede and oilseed rape) and also radish and ornamental and spontaneous crucifers. Nevertheless, there is considerable variation within the pathogens and, so far, 11 races have been described for this pathogen – these races can be differentiated through inoculation of a series of differential brassicas. Races 1 and 4 seem to be the most frequent races identified in *Brassica oleracea* crops (e.g. cauliflower, cabbage, kale, broccoli) worldwide. Other races including races 3, 5 and 6 have also been identified in the UK, but appear to be less frequent.

An AHDB project, FV 186, on black rot of cauliflower crops in Cornwall, completed in 1998 showed that infection was present in over 20% of the seed lots tested although the levels were generally low, and two races,1 and 4, were identified, with race 1 being predominant.

An outbreak of black rot was observed in the first trimester of 2019 in cauliflower crops in Cornwall. Growers indicated that they would like to know more about this outbreak, including the race-type of bacteria causing it, and to try to establish if the isolates were similar to isolates collected in the past.

Summary

Samples of leaves from three cauliflower cultivars grown in Cornwall, showing necrosis and chlorosis that could be attributed to black rot of crucifers, were received in March 2019.

Isolations were performed from the edge of the lesions and ten isolates of potential *Xanthomonas campestris* pv. *campestris* (Xcc) were kept for further characterisation.

The ten isolates from Cornwall were race-typed in glasshouse conditions at the School of Life Sciences, Wellesbourne Campus, University of Warwick, alongside control isolates of known races including two isolates previously obtained from Cornwall. Two isolates that represent two new races (10 and 11) were also tested in the same experiment.

The isolates from Cornwall belong to two races, 1 and 4, with race 4 being predominant in the tested samples. The isolates were very aggressive in cauliflower and in several other brassicas and some systemic infections were observed.

One isolate from Portugal of a previously proposed race 10, behaved like race 4 when inoculated in the differential set of brassicas and another Portuguese isolate previously identified as race 11, might not be different from race 6 isolates. This indicated that more research will be needed to test all isolates of proposed new races, to be able to confirm their existence.

The isolates from Cornwall and a range of other *X. campestris* isolates were tested by PCR with primers targeting *X. campestris*, *X. campestris* pvs *campestris* (Xcc) and *raphani* (Xcr) and Xcc races 1 and 4. All isolates from Cornwall were confirmed as *X. campestris*. The results indicated that the Xcc pathovar-specific primers might not detect all Xcc isolates and Xcr primers might detect some other pathovars. Assays with race-specific primers for race 1 correctly identified the race 1 isolates, but primers for race 4 seem to lack specificity as they detected some isolates of other races. Testing of existing tests for detection of pathovars and races and development of better tests will be important for quick detection of specific pathovars and races.

Financial Benefits

N/A

Action Points

Currently there are no cultivars of cauliflower nor other *B. oleracea* crops available with resistance to races 1 and 4 of black rot and there are no chemicals approved for use against this disease. Therefore, good hygiene measures should be applied as detailed in the recently updated AHDB Factsheet (Roberts, 2019). In particular, growers should:

- Ensure that starting material, seeds and transplants are free from *Xanthomonas*
- Avoid use of overhead irrigation as it has been shown to increase spread of the disease in plant nurseries
- Remove all waste from infected brassicas from the fields and consider doing rotations after outbreaks of the disease
- Consider that ornamentals like wallflower or different crops like swede (*B. napus*) could have different races of Xcc that could transfer to vegetable *B. oleracea* crops like cauliflower and cabbage

Breeding for resistance should aim at introducing resistance to races 1 and 4 into commercial vegetable brassicas, but other races should also be considered including races 3, 5 and 6 that have also been shown to be present in the UK.

SCIENCE SECTION

Introduction

Black rot of crucifers is one of the most important diseases of brassicas worldwide (Williams, 1980; Vicente and Holub, 2013). The disease is favoured by warm and humid conditions and in some climates can lead to total loss of brassica production. This disease occurs regularly in UK vegetable brassica crops, but its importance tends to vary from year to year possibly due to different levels of contamination of seeds and transplants and variable weather conditions.

Control of this disease relies mostly on testing seeds and good hygiene practices in the nurseries and fields. A recent update of an AHDB factsheet on black rot of crucifers summarises the current advice to Growers (Roberts, 2019). There are no resistant varieties of the main vegetable brassicas available although some levels of partial resistance/tolerance to some isolates have been reported (Vicente et al., 2002).

Black rot of crucifers is caused by the bacterium *Xanthomonas campestris* pv. *campestris*. (Xcc). Bacteria generally enter the plants through the hydathodes (located around the leaf margins) and cause typical V-shape lesions and dark veins when the bacterial colonies start blocking the plant vascular system. Different conditions, different bacterial isolates and different hosts can cause symptoms that vary from the typical – more necrosis that can appear dry or papery blight can occur and, in some conditions, leaf spots have been observed, but generally they then tend to evolve to V-shape symptoms as the season progresses.

Other closely related pathovars can also infect brassica crops including *X. campestris* pv. *raphani* (Xcr) that causes a non-vascular leaf spot disease of crucifers and tomato (Vicente et al., 2006). This pathovar has been identified in the UK in wallflower (Stead et al., 2016), but so far, it does not appear to be causing significant damages in brassica crops in the UK, but it seems to be more important in certain US areas (Lange et al., 2016). In addition, *X. campestris* pv. *incanae* is a pathogen of garden stocks and does not seem to cause disease in vegetable brassica crops (Vicente et al., 2001).

All brassicas can be infected by Xcc including the vegetable brassicas from different species and also radish, ornamental crucifers and weeds. Nevertheless, there is considerable variation within the pathogens and up to now 11 races have been described for this pathogen – these races can be differentiated through inoculation of a series of differential brassicas (Table 1). Races 1 and 4 have been the most frequent races identified in *Brassica oleracea* crops (e.g. cauliflower, cabbage, kale, broccoli) in the UK and worldwide (Vicente et al., 2001;

Mulema et al. 2011, Singh et al. 2016, Bella et al., 2019). Other races including races 3, 5 and 6 have also been identified in the UK (Vicente et al., unpublished).

TABLE 1. Races and postulated avirulence genes of *Xanthomonas campestris* pv. *campestris* (Xcc) and matching genes of brassicas. Adapted from Fargier and Manceau (2007), Vicente and Holub (2013) and Cruz et al. (2017).

		Races / Postulated avirulence genes (A)										
Differential cultivars or accessions	**Postulated resistance genes (R)**	1	2	3	4	5	6	7	8	9	10	11
		A1	.	A1	A1	.	.	.		A1	.	.
		.	A2	.	.	.	.	.		.	.	.
		.	A3	A3	.	A3	.	.		A3	.	.
		.	.	.	A4	.	.	.	.	A4	.	.
		.	.	.	.	.	.	A5	.	.	A5	.
		.	.	.	.	.	.	.	.	.	A6	A6
Wirosa F_1 *(B. oleracea*)	.	+	+	+	+	+	+	+	+	(+)	+	+
Just Right Hybrid Turnip *(B. rapa)* Selection of Cobra COB60 (*B. napus*)	R4	+	+	+	-	+	+	+	-	(+)/+	+	+
Seven Top Turnip *(B. rapa)*	R2 R4 R6	+,v	-	+,v	-,v	+,v	+	+	-/(+)	-/(+)	-	-
Selection of PI199947, PIC1 (*B. carinata*)	R1 R4?	-	(+)	-	-	+	+	+	-	-	+	+
Florida Broad Leaf Mustard, FBLM2 (*B. juncea*)	R1 R5	-	+	-	-	(+)	+	-	-	-	-	+
Miracle F_1 *(B. oleracea),* SxD1 (*B. oleracea*)	R3	+	-/(+)	-	+	-	(+)/+	+	-/(+)	(+)	+	(+)/+

Black rot symptoms including V shape chlorotic and necrotic marginal lesions: +, compatible interaction (susceptibility); -, incompatible interaction (resistance); (+), weakly pathogenic.
nt, not tested; v, variable; ? indicates that the gene might be present, although might not be necessary to explain the interaction.

An AHDB project on black rot in cauliflower crops of Cornwall (FV 186) was completed over 20 years ago, in 1998. This study showed that infection was present in over 20% of the seed lots tested although the levels were generally low, and two races 1 and 4 were identified, with race 1 being predominant (Roberts, 1998).

An outbreak of black rot was observed in the first trimester of 2019 in cauliflower crops in Cornwall and it was felt that it would be useful to find the races of Xcc causing the symptoms and to compare this outbreak with the information previously obtained for this region.

The diagnostics of Xcc can be speeded through the use of molecular methods and in particular the use of standard PCR with primers targeting genes or particular genomic regions of *X. campestris*. The ISTA protocols recommend a multiplex PCR with the primers of Rijlaarsdam et al. (2004) that target the Zup gene and Berg et al. (2005) that target hrpF gene together with universal primers (Roberts and Koenraad, 2014). Primers targeting pathovar *campestris* and pathovar *raphani* have been developed by Leu et al. (2010). Recently primers targeting specific races have been developed (Rubel et al., 2017) but these primers need to be validated using large sets of isolates representing different races and pathovars.

The aims of this project were:

- to characterise the isolates obtained from the outbreak in Cornwall in 2019 through race-typing using a standard brassica differential series including comparisons with UK isolates obtained from other outbreaks
- to test isolates representative of two recently described Xcc races (races 10 and 11)
- to test the isolates from Cornwall and a range of isolates through PCR with primers targeting *X. campestris*, pathovars *campestris* and *raphani* and two specific races (1 and 4) of Xcc to assess the usefulness of these primers for identification of isolates

Materials and methods

Samples of leaves from three cauliflower cultivars grown in Cornwall, showing necrosis and chlorosis that could be attributed to black rot of crucifers, were received on 15 March 2019 (Appendix Figures 1 and 2).

Isolations were performed from leaf samples, from the edge of lesions. In short, small portions of leaf (rectangles 4 mm x 2 mm) were macerated in 400 ml of sterile distilled water and several loop-fulls of the suspension were spread in petri dishes with Kings' B (KB) growing medium. Plates were sealed with parafilm and incubated for 72 hrs at 28°C. Single colonies were transferred to KB and YDC plates for observation of morphology. Isolates that appeared pure and uniform, were frozen in duplicate tubes with nutrient broth and glycerol and stored in a freezer at -76°C as described in Vicente et al. (2001).

Race-typing was performed using eight lines of Brassica spp. included in Table 1. Seeds were sown in 7 cm square pots with M2 compost in a glasshouse at the Wellesbourne Campus in May 2019. Two or three seeds were sown per pot. One pot with two or three plants of each accession and at least two pots of Seven Top Turnip were inoculated with each isolate. Ten isolates obtained from the 2019 samples from Cornwall were race-typed together

with control isolates from races 1, 4, 5, 6 and 7 (Table 2). In addition, two isolates received from the Colecção Portuguesa de Bactérias Fitopatogénicas (CPBF) previously reported as belonging to two new races 10 and 11 (Cruz et al., 2017), were also race-typed in duplicate number of plants.

The bacterial isolates were recovered from cultures stored at -76°C and grown on KB agar plates at 28°C for 48 h. Three young leaves of four-week-old plants were inoculated using mouse-tooth forceps dipped in a bacterial suspension and the presence of symptoms was recorded two and three weeks after inoculation according to the methods previously developed for inoculation of brassicas (Vicente et al., 2001) (Appendix Figures 3 and 4).

Table 2. Isolates used in the current study for race-typing and PCR experiments

WHRI Number	Other information	Host of origin	Location	Year of isolation	Reference
Isolates from Cornwall, 2019					
10018A		Cauliflower, Var1	Cornwall, UK	2019	This study
10018B		Cauliflower, Var1	Cornwall, UK	2019	This study
10018C		Cauliflower, Var1	Cornwall, UK	2019	This study
10019A		Cauliflower, Var2	Cornwall, UK	2019	This study
10019B		Cauliflower, Var2	Cornwall, UK	2019	This study
10019C		Cauliflower, Var2	Cornwall, UK	2019	This study
10020A		Cauliflower, Var3	Cornwall, UK	2019	This study
10020B		Cauliflower, Var3	Cornwall, UK	2019	This study
10021A		Cauliflower, Var3	Cornwall, UK	2019	This study
10021B		Cauliflower, Var3	Cornwall, UK	2019	This study
Xanthomonas campestris pv. *campestris* (Xcc) isolates previously obtained from Cornwall					
1279A	Race 4 type	Cauliflower	Cornwall, UK	1984	Vicente et al. (2001)
3818A	Race 1	Cauliflower	Cornwall, UK	1994	Vicente et al. (2001)
Other Xcc control isolates					
8857*	Race 1	Kale	Ovar, Portugal	2014	Vicente (Unpublished)
8917A*	Race 4	Kale	Furad., Portugal	2014	Vicente (Unpublished)
8979	Race 5	Cauliflower	Lincolnshire, UK	2017	Vicente (Unpublished)
8960	Race 6	Wallflower	Essex, UK	2016	Vicente (Unpublished)
8450A	Race 7 type	Cabbage	Dhankuta, Nepal	2001	Jensen et al. (2010)
Possible new Xcc races (received as races 10 and 11 respectively)					
10023A,B	CPBF 331	Tronchuda cabbage	Sintra, Portugal	2004	Cruz et al. (2017)
10024A,B	CPBF 1198	Wild mustard	Lisbon, Portugal	2007	Cruz et al. (2017)
Negative control (non-Xanthomonas)					
10025A		Kale	Furad., Portugal	2019	Vicente (Unpublished)
Other *X. campestris* pathovars: *incanae* (Xci), *raphani* (Xcr) and *X. campestris* (Xc) from ornamental candytuft (Iberis)**					
8474	Xcr	Wallflower	Sussex, UK	1990	Stead et al. (2016)
8305	Xcr	Turnip greens	Oklahoma, US	1995	Vicente et al. (2006)
6377	Xci	Stocks	US	1950	Vicente et al. (2006)
6375	Xc	Candytuft	Tanzania	1954	Vicente et al. (2006)

* Race-typed previously. Used for PCR tests.
**Used only for PCR with primers from Leu et al. (2010)

For molecular analyses, DNA extraction was performed using a Bioline Isolate II extraction kit following the manufacturer's protocol for extraction of DNA from bacteria.

Polymerase Chain Reaction (PCR) with a range of primers targeting *Xanthomonas campestris*, pathovars *campestris* and *raphani* and races 1 and 4 of *Xanthomonas campestris* were performed for amplification of the target regions.

All primers listed in Table 3 were synthesised by Sigma-Aldrich (Dorset, UK) and diluted to 10 µM. Negative controls without DNA template were included in all PCR runs. Reactions were conducted in 10 µl volume containing 0.2 µl of template DNA solution (approximately 10 ng of bacterial DNA), forward and reverse primers (0.4 µL each at 10 µM), 5x Hi-Fi buffer (2 µL), dNTPs (1 µL at 10 mM), water (5.8 µL) and enzyme Velocity (0.2 µL). The reactions were done in a GeneAmp PCR System 9700 (Applied BioSystems) thermal cycler.

Table 3. Primers tested in the current study

Number	Original designation	Length/ Temp.	Sequence	Target gene	Annealing temperature / Expected band size (bp)
To detect *Xanthomonas campestris* (Berg et al., 2005)					
JV51	DLH120	20	CCGTAGCACTTAGTGCAATG	hrpF	58°C / 619
JV52	DLH125	22	GCATTTCCATCGGTCACGATTG		
To detect *Xanthomonas campestris* pv. *campestris* (Leu et al., 2010)					
JV53	Xcc 2f	20	TGGGTTTTCGCCTATCAAAC	Unknown	60°C / 200
JV54	Xcc 2r	20	TGCAACTATTCCTAGCACCG		
To detect *Xanthomonas campestris* pv. *raphani* (Leu et al., 2010)					
JV55	Xcr 14f	20	CGTTAGCCAGGTAGAAAGCG	Unknown	60°C / 277
JV56	Xcr 14r	20	TCGCTATTTCCATCTACCCG		
To detect *Xanthomonas campestris* pv. *campestris* race 1 (Rubel et al., 2017)					
JV43	Xcc_47R1_F	22	CCTCCTGAGTCATGGCAATGGC	xcc-b100_4389	65°C / 1089
JV44	Xcc_47R1_R	21	TAGCAGGGGAGTGCTGCTTGC		
JV45	Xcc_85R1_F	22	GCGGCTCGGCTTCACGGTCAGC	xcc-b100_4275	65°C / 467 (other races 872)
JV46	Xcc_85R1_R	21	GCCCAGGATGCAGCGCAGCGT		
To detect *Xanthomonas campestris* pv. *campestris* race 4 (Rubel et al., 2017)					
JV47	Xcc1_46R4_F	22	GGCATGGGGAATGATCGTTGAC	Intergenic	67°C / 462
JV48	Xcc1_46R4_R	21	ATGCGGGCGATGGGATGGCCA		
JV49	Xcc2_46R4_F	22	GCGTAGCGAAAACTGGTAGTTC	Intergenic	67°C / 578
JV50	Xcc2_46R4_R	23	GCACAGGCGCACCAGCATATGGC		

The conditions for amplification for initial testing of all primers were: denaturation at 98˚C for 3 min followed by 35 cycles of amplification at 98˚C for 30 s, annealing at 58˚C for 30 s and 72˚C for 1 min, and terminated by a final elongation at 72˚C for 8 min.

Additionally, PCRs with primers JV53/54 and JV55/56 (Leu et al., 2010) with DNA from 10 isolates including two Xcr isolates, and *X. campestris* pv. *incanae* and a *X. campestris* isolate from the ornamental candytuft (*Iberis* sp.) were performed with the following conditions:

denaturation 98°C for 3 min, 35 cycles of denaturation at 98°C for 30s, annealing at 60°C for 30s and extension at 72°C for 30s, followed by a final extension at 72 °C for 5 min.

PCR with primers targeting races 1 and 4 (Rubel et al., 2017) were repeated with the following conditions: denaturation 98°C for 3 min, 35 cycles of denaturation at 98 °C for 30s, annealing at 65°C (for race 1) and 66°C (for race 4) for 30s and extension at 72°C for 1 min, followed by a final extension at 72°C for 5 min.

Purity of the PCR products was checked by running 5 µl reaction mixture on a 1% agarose gel stained with GelRed™ nucleic acid gel stain (Biotium Inc. Hayward, CA, USA) by electrophoresis for 1 hour at 120V and the gels were visualised and photographed on an UV transilluminator and a G:Box gel imaging system (Syngene, Cambridge, UK).

Results

Ten isolates were obtained from three varieties of cauliflower leaves received from Cornwall in March 2019. All isolates were given unique numbers/letters and were preserved in the WHRI collection located at the Wellesbourne Campus, School of Life Sciences, University of Warwick.

Results of race-typing of the 2019 isolates from Cornwall as well as isolates of different races used for comparison are presented in Table 4. Eight isolates were identified as race 4 and two isolates as race 1 with similar results to isolates obtained previously from Cornwall (WHRI 3818A and 1279A) (Appendix Figures 5 and 6). The isolates from Cornwall were very aggressive on cauliflower and systemic infections of the plants were observed especially in plants inoculated with race 1 isolates (Appendix Figures 7 and 8).

An isolate received from Portugal as a new race 10 (CPBF 331) had the pattern of reaction similar to the race 4 isolates and therefore we could not confirm the results of Cruz et al. (2017). The differences between races 4 and 10 as reported by Cruz et al. (2017) are the reactions of Just Right Turnip (JRT) and Seven Top Turnip (STT). The reaction of JRT and COB60 were clearly negative in our experiment and most STT plants were resistant although a small number were susceptible confirming that this cultivar is variable and should not be taken as the basis for establishing new races. Table 5 presents the results obtained with multiple plants inoculated with Seven Top Turnip.

An isolate received from Portugal as a new race 11 (CPBF 1198) had a pattern similar to race 6. The difference between races 6 and 11 according to Cruz et al. (2017) is only the reaction of STT and the variability of this accession can cause confusion in the assessment.

The isolates used as controls for races 5, 6 and 7 behaved as expected. Importantly the results with race 6 isolate WHRI 8960 also showed variability in STT.

TABLE 4. Results of race-typing ten isolates from Cornwall obtained in this study together with isolates from different races, two isolates received from Portugal as new races 10 (WHRI 10023A,B) and 11 (WHRI 10024A,B) and a non-Xanthomonas isolate (used as negative control)

		Origin (Year) / Host of origin / WHRI isolate number									
		Cornwall (1994)	Cornwall (2019)	Cornwall (1984)	Cornwall (2019)	Lincolnshire (2017)	Essex (2016)	Nepal (2001)	Portugal (2004)	Portugal (2007)	Portugal (2019)
		Cauliflower	Cauliflower	Cauliflower	Cauliflower	Cauliflower	Wallflower	Cabbage	Tronchuda cabbage	Wild mustard	Kale
Differential cultivars or accessions	**Species**	3818A	10018A 10021B	1279A	10018B 10018C 10019A 10019B 10019C 10020A* 10020B 10021A	8979	8960	8450A	10023A 10023B	10024A 10024B	10025A
Wirosa F_1	*B. oleracea*	+	+	+	+	+	+	+	+	+	-
Just Right Turnip	*B. rapa*	+	+	-	-	+	+	+	-	+	-
COB60	*B. napus*	+	+	-	-	+	+	+	-	+	-
Seven Top Turnip	*B. rapa*	+	+, -, v	-, +, v	-, +, v	+	-, +, v	+	-, +, v	-, +, v	-
PIC1	*B. carinata*	-	-	-	-	+	+	+	-	+	-
FBLM2	*B. juncea*	-	-	-	-	+	+	-/(+)	-	+	-
Miracle F_1	*B.oleracea*	+	+	+	+	-/(+)	+	+	+	+	-
SxD1	*B. oleracea*	+	+	+	+	-	+	+	+	+	-
RACE		1	1	4	4	5	6	7	4	6	Not path.

Black rot symptoms including V shape chlorotic and necrotic marginal lesions: +, compatible interaction (susceptibility); -, incompatible interaction (resistance); (+), weakly pathogenic.
nt, not tested; v, variable.
*WHRI 10020A showed some lesions on Just Right Turnip, but not on COB60.

Table 5. Reaction of plants of Seven Top Turnip (STT) with isolates from two races of *Xanthomonas campestris* pv. *campestris*

	Number of plants inoculated with each isolate			
	10023A, B (race 4)	1279A (race 4 control)	10024A, B (race 6)	8960 (race 6 control)
Resistant	32	5	14	3
Susceptible	3	1	25	5
Total tested	35	6	39	8

The results of PCR performed with seven pairs of primers targeting *Xanthomonas campestris*, *X. campestris* pvs. *campestris* (Xcc) and *raphani*, and races 1 and 4 of Xcc are presented in Table 6.

All isolates tested were positive with primers DLH120/DLH125 (Berg et al., 2005) that target *X. campestris* except for the non-Xanthomonas isolate WHRI 10025A that produced a larger band.

All isolates were positive with primers from Leu et al. (2010) targeting Xcc with the exception of WHRI 8979, and all isolates were negative with primers targeting Xcr. Additional PCRs were performed with these two sets of primers including Xcr, Xci and an isolate from the ornamental candytuft (Table 6). Primers targeting Xcc also amplified a similar size band from one Xcr isolate and the isolate from candytuft. Primers targeting Xcr amplified the 277 bp product from two Xcr isolates, but also the *X. campestris* pv. *incanae* and the isolate from candytuft (Appendix Figure 9 and 10).

PCRs with primers targeting Xcc race 1, allowed the correct identification of the four Xcc race 1 isolates included in this study (Table 6). The second set of primers (JV45/46) produced strong bands only with race 1 isolates (Appendix Figure 11).

PCRs with primers targeting Xcc race 4, did not differentiate the race 4 isolates from all others (Table 6). The first set of primers produced a large band with isolate WHRI1279A that was used to design the primers, but not with other race 4 isolates; a range of isolates had a smaller strong band (Figure 10). The second set of primers produced many non-specific bands, but seemed to produce a different profile for isolate WHRI1279A (Appendix Figure 12).

Table 6. Results of PCR with targeting *Xanthomonas campestris*, *X. campestris* pvs *campestris* and *raphani*, and races 1 and 4 of *X. campestris* pv. *campestris*

WHRI Number	Race-type	Primer pairs / target / band size						
		DLH120/ DLH125	Xcc_2f/ Xcc_2r	Xcr_4f/ Xcr_14r	Xcc_47R 1F/ Xcc_47R 1R	Xcc_85R 1_F/ Xcc_85R 1_R	Xcc1_46_ R4F/ Xcc1_46 R4_R	Xcc2_46 R4_F/ Xcc2_46_ R4_R
		Xc	Xcc	Xcr	Xcc race 1	Xcc race 1	Xcc race 4	Xcc race 4
		619bp	200bp	277bp	1089bp*	467bp	462bp**	578bp***
Isolates from Cornwall, 2019								
10018A	1	+	+	-	(+)	+	+	-
10018B	4	+	+	-	-	-	+	-
10018C	4	+	+	-	-	-	+	-
10019A	4	+	+	-	-	-	+	-
10019B	4	+	+	-	-	-	+	-
10019C	4	+	+	-	-	-	+	-
10020A	4	+	+	-	-	-	+	-
10020B	4	+	+	-	-	-	+	-
10021A	4	+	+	-	-	-	+	-
10021B	1	+	+	-	(+)	+	+	-
Isolates previously obtained from Cornwall								
1279A	4	+	+	-	-	-	+++	(+)
3818A	1	+	+	-	(+)	+	-	-
Other control isolates								
8857	1	+	+	-	(+)	+	+	-
8917A	4	+	+	-	-	-	+	-
8979	5	+	-	-	-	-	+	-
8960	6	+	+	-	-	-	+	-
8450A	7	+	+	-	-	-	++	-
Received as new races (10 and 11 respectively)								
10023B	4	+	+	-	-	-	+	-
10024B	6	+	+	-	-	-	+	-
Negative control (non-*Xanthomonas*)								
10025A	np	- (bigger band)	-	-	-	-	-	-
Other *X. campestris* pathovars								
8474	Xcr	nt	-	+	nt	nt	nt	nt
8305	Xcr	nt	+	+	nt	nt	nt	nt
6377	Xci	nt	-	+	nt	nt	nt	nt
6375	Xc	nt	+	+	nt	nt	nt	nt

+, band present; (+) band present, but not easy to differentiate; nt, not tested
*Profiles show unspecific bands; results of a smaller band
**Some strong bands of different sizes
** *Profiles show many unspecific bands; results of presence of a particular band differentiate WHRI1279A

Discussion

Black rot of crucifers is a regular occurrence in vegetable brassica crops in the UK and in some years, it causes economical loses in brassica producing regions including Cornwall and Lincolnshire. Isolates obtained from cauliflower leaf samples grown in Cornwall in 2018/19 were race-typed using a brassica differential series and were shown to belong to two different

Xcc races, 1 and 4. This was similar to the conclusion from race-typing performed over 20 years ago as part of the AHDB project FV 186 (Roberts, 1998), although race 4 was predominant in the samples from 2019 whilst race 1 was predominant in samples from the late 90s. Nevertheless, the number of isolates obtained is too limited to reach a conclusion regarding the abundance of races and a larger study would have to be conducted to ascertain abundance of each race.

Races 1 and 4 have also been reported as the most frequent races of vegetable *B. oleracea* crops in other regions of the world, including European countries like Italy (Bella et al., 2019), African countries like Kenya (Mulema et al, 2012) and in India (Singh et al., 2016) and Nepal (Jensen et al., 2010).

Other races have been identified in the UK recently including race 3 in wild brassicas (Passo, 2016), race 5 in cabbage and swede and race 6 in wild turnips and wallflower (Vicente et al., unpublished). The type-strain of Xcc is also a race 3 isolate obtained in the UK in 1957 (Vicente et al., 2001).

We could not confirm the pattern of reaction of isolates received as new Xcc races 10 and 11. Therefore, it would be important to test the other isolates from Cruz et al. (2017) to assess if they conform to the pattern proposed for these new races.

As no resistant cultivars of the main brassica crops are available (e.g. cauliflower, cabbage, broccoli), growers should continue to follow recommendations for ensuring that they start with healthy material (seeds and transplants) and put in practice good hygiene practices as highlighted in Roberts (2019).

Future breeding for resistance in *B. oleracea* (e.g. cauliflower and cabage) that would be useful for the UK production should target races 1 and 4, but also consider races 5 and 6 as these races are present and could increase in importance if crops resistant only to races 1 and 4 were deployed. Resistance to races 1 and 4 is very rare in *B. oleracea* crops and it is possible that useful resistance should have to be introgressed from other crops like turnips or mustards where resistance to these races is more common (Taylor et al., 2002). Resistance to races 3 and 5 and quantitative or partial resistance to race 6 might exist in the *B. oleracea* genome.

Inoculating plants to identify Xcc (and related pathovars) and for determining Xcc races is a time-consuming process that can take nearly two months from sowing. Using PCR to detect *X. campestris*, the different pathovars (including *campestris*, *incanae* and *raphani*) and different races, could speed the process considerably and reduce the cost of testing.

PCRs with primers targeting the hrpF gene (Berg et al., 2005) produced a positive result will all tested Xcc isolates as predicted. These primers are used routinely and they can be useful for the identification of *X. campestris*. Nevertheless, tests with these primers do not differentiate pathovars (*campestris*, *raphani*, *incanae*) and they also have been shown to be positive with *X. hortorum* pv. *carotae* (Eichmeier et al., 2019).

PCRs with primers developed by Leu et al. (2010) targeting Xcc worked with all DNA samples from Xcc isolates except isolate WHRI 8979. These PCRs were also positive with one Xcr isolate and the isolate from ornamental candytuft. This shows that there will be exceptions and there could be some false negative and positive results using this primer set.

PCRs with primers targeting Xcr (Leu et al., 2010) were negative for all Xcc isolates, but were positive with Xcr, Xci and an isolate from the ornamental Iberis. Therefore, this primer pair is not specific for pathovar *raphani*. Rubel et al. (2019) also showed that this primer pair produces positive results for Xci. These authors have designed new primers for pathovar *raphani*, but these primers will need to be tested with a larger collection of isolates to check their specificity.

PCRs with primers targeting races 1 and 4 (Rubel et al., 2017) showed that the primers for race 1 might indeed target all or a group of Xcc race 1, but primers for race 4 did not allow the identification of all the race 4 isolates tested. These primers were designed based on a very restricted number of sequences and this can lead to assays that lack specificity and/or that only detect a small number of isolates (in this case, WHRI1279A). It will be important to obtain more sequences of race-typed and well characterised isolates in order to design better diagnostic primers for the different races in the near future. Using race-specific primers could speed the characterisation of new isolates from UK outbreaks as only a selection of isolates would need to be tested in differential brassicas; the information gathered should be relevant to establish control strategies including future breeding programmes.

Conclusions

- The 10 isolates obtained from samples of cauliflower from Cornwall caused typical black rot symptoms in a range of brassicas and were shown to belong to two different races, 1 and 4, with race 4 being predominant
- The pattern of reaction of new races 10 and 11 could not be confirmed for two isolates tested
- Although this study included a limited number of samples/isolates, races 1 and 4 are probably still the most frequent races in vegetable brassicas in the UK and future breeding for resistance should target these races

- Other races have been identified in the UK including races 3, 5 and 6
- PCR testing of the isolates from Cornwall and control isolates with primers targeting the hrpF gene confirmed that they are *Xanthomonas campestris*
- PCR testing with two sets of pathovar specific primers showed that these primers are inadequate to differentiate the *X. campestris* pathovars
- PCR with primers targeting race 1 detected all race 1 isolates included in this study, but PCR with primers targeting race 4 were not specific enough
- Testing isolates in susceptible and differential brassicas is still recommended to determine the pathogenicity and race-type, but in the future better molecular markers might allow to speed the process of pathovar and race determination

Knowledge and Technology Transfer

Results from this project have been presented by Joana G. Vicente as part of oral communications at the following events:

- UK Brassica Research Community, 5 June 2019, University of Warwick
- Third Annual Conference of COST Action EuroXanth, 9-12 September 2019, Lednice, Czech Republic

Results from this project will be presented by Joana G. Vicente at the Brassica Growers Association R&D Meeting, 8 October 2019, Elsoms Seeds, Spalding.

Glossary

Differential brassica plant: line or cultivar that shows a different reaction (susceptible or resistant) with different isolates of Xcc and therefore contributes to the differentiation of races

PCR: Polymerase Chain Reaction

Xcc: bacterial plant pathogen *Xanthomonas campestris* pv. *campestris* the cause of black rot of crucifers

Xci: bacterial plant pathogen Xanthomonas campestris pv. incanae the cause of

Xcr: bacterial plant pathogen Xanthomonas campestris pv. raphani, the cause of a leaf spot disease of crucifers

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Appendices

Figure 1. Cauliflowers in Cornwall showing symptoms of black rot caused by *Xanthomonas campestris* pv. *campestris* in 2019 (photos by Dawn Teverson)

Figure 2. Some of the leaf samples of cauliflower from which isolations were performed

Figure 3. Four-week-old plants ready for inoculations in the glasshouse at the Wellesbourne Campus, School of Life Sciences, University of Warwick

Figure 4. Method of inoculation using mouse-tooth forceps wrapped in cotton wool dipped in a suspension of bacteria

Figure 5. General view of inoculated plants in the glasshouse

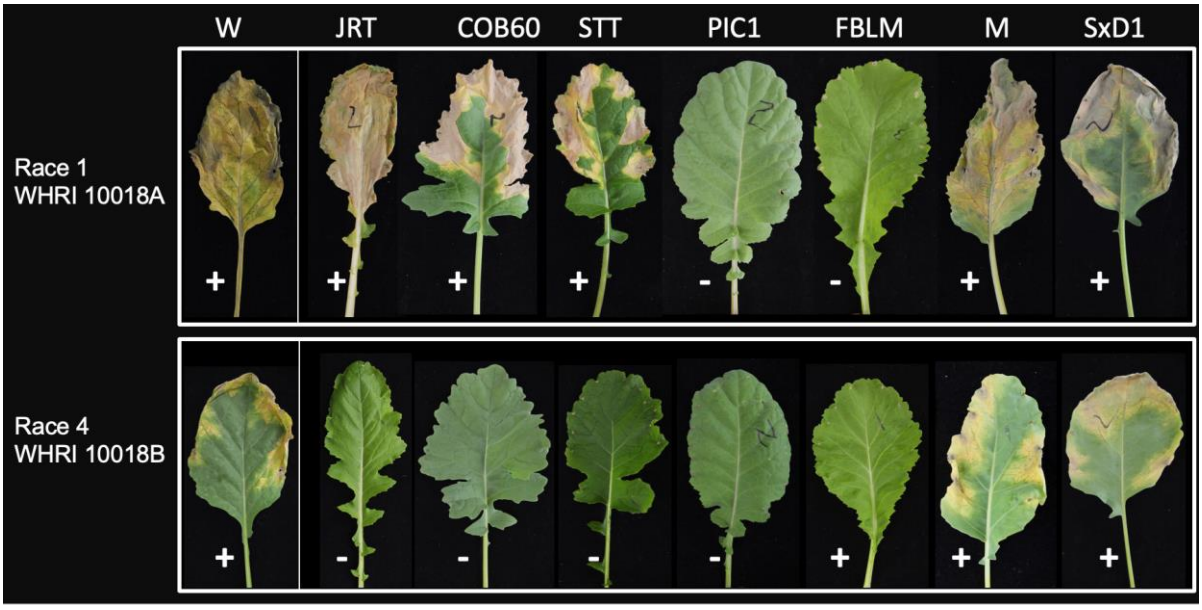

Figure 6. Pattern of reaction of leaves plants inoculated with isolates of races 1 (WHRI 10018A) and 4 (WHRI 10018B) from Cornwall

Figure 7. Cauliflower cv. Miracle F_1 after inoculation with race 1 isolate WHRI 10018A showing symptoms of systemic infection with wilting of leaves that were not inoculated

Figure 8. Uninoculated leaf of cauliflower cv. Miracle F_1 showing typical symptoms of systemic infection after other leaves were inoculated with race 1 isolate WHRI 10018A

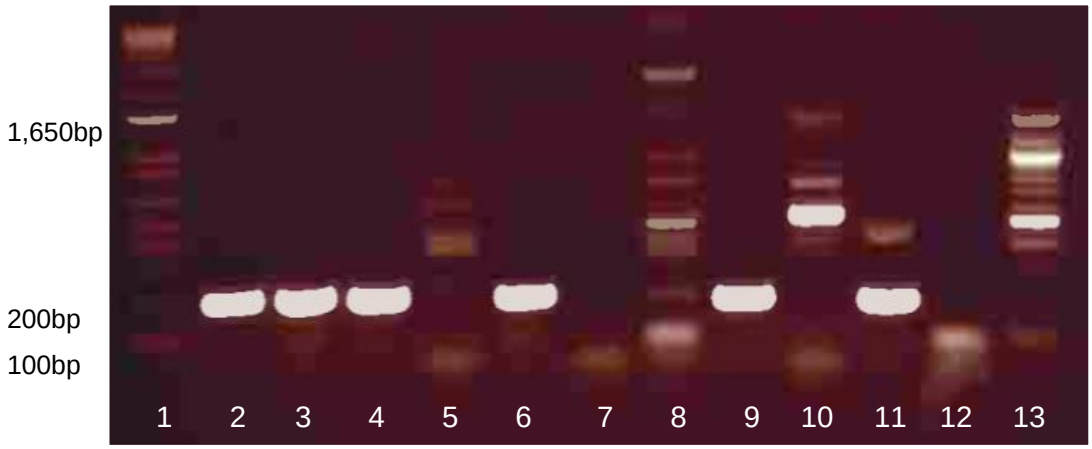

Figure 9. Gel showing results of PCRs with primers targeting Xcc (Leu et al., 2010). Lanes as follow:
Lanes as follows (WHRI numbers):

1: 1kb plus ladder	2: 10018A (Xcc race 1)	3: 10019A (Xcc race 4)	4: 8857 (Xcc race 1)
5: 8979 (Xcc race 5)	6: 8960 (Xcc race 6)	7: 10025A (non-Xanth.)	8: 8474 (Xcr)
9: 8305 (Xcr)	10: 6377 (Xci)	11: 6375 (Xc Iberis)	12: Negative control
13: 100bp ladder			

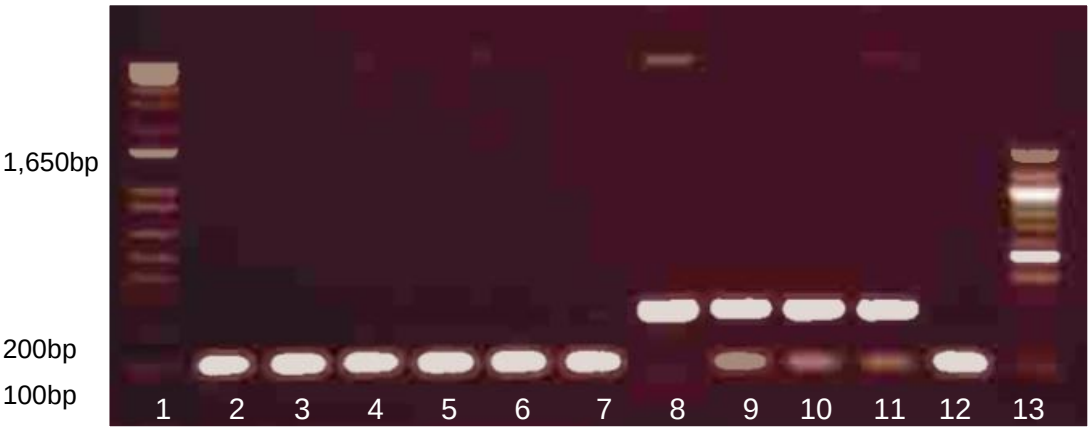

Figure 10. Gel showing results of PCRs with primers targeting Xcc (Leu et al., 2010). Lanes as in Figure 9

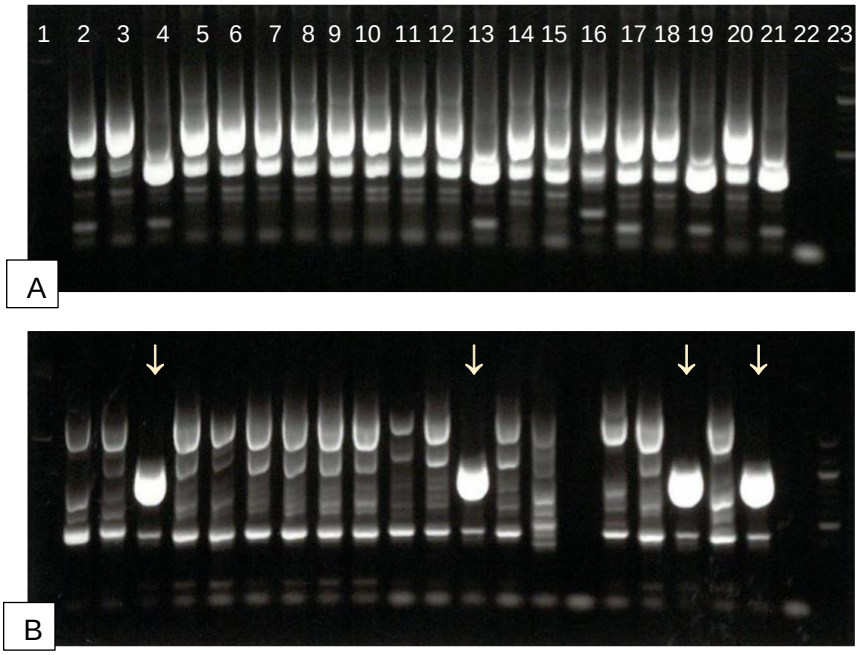

Figure 11. Gel showing results of PCRs with primers targeting Xcc race 1 (Rubel et al., 2017). A, primers (JV43/44) and B, primers (45/46). Arrows indicate the race 1 isolates. Lanes as follows (WHRI number and race):

1: 1kb plus ladder	2: 8960, race 6	3: 8979, race 5	4: 10018A, race 1
5: 10018B, race 4	6: 10018C, race 4	7: 10019A, race 4	8: 10019B, race 4
9: 10019C, race 4	10: 10020A, race 4	11: 10020B, race 4	12: 10021A, race 4
13: 10021B, race 1	14: 10023B, race 4	15: 10024B, race 6	16: 10025A (non-pathogen)
17: 1279A, race 4	18: 8817A, race 4	19: 8857, race 1	20: 8450A, race 7
21: 3818A, race 1	22: Negative control	23: 100bp ladder	

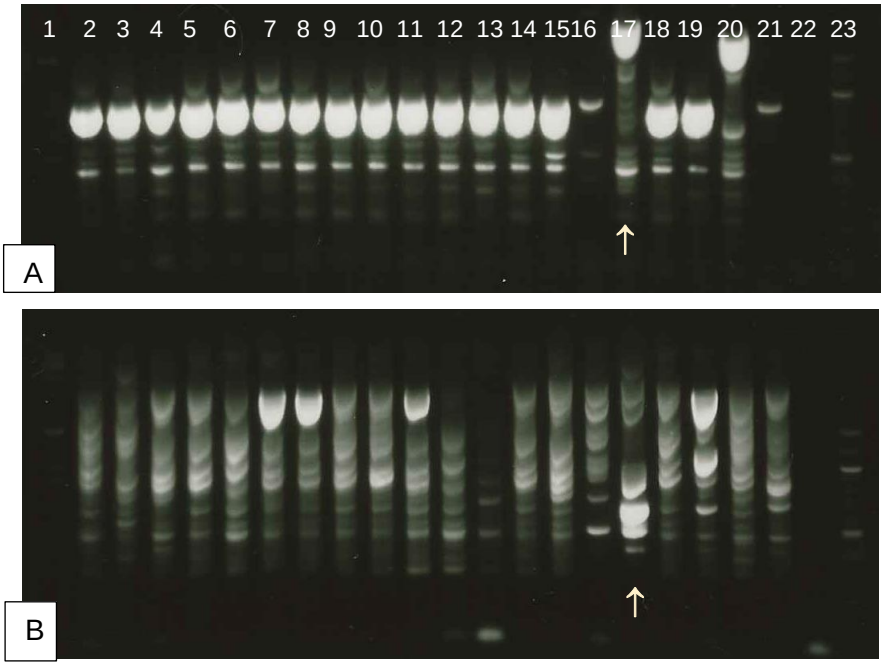

Figure 12. Gel showing results of PCRs with primers targeting Xcc race 4 (Rubel et al., 2017). A, primers (JV47/48) and B, primers (49/50). Arrow indicate the race 4 isolate WHRI1279A. Lanes as in Figure 11