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Improved management of black rot of brassicas

Len Tesoriero
NSW Department of Primary Industries

Project Number: VG01024

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FINAL REPORT

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Quality assurance for improved management of black rot of Brassicas: improved detection and disinfestation in seed; management protocols for seedling and field production

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NSW Agriculture

NSW DEPARTMENT OF PRIMARY INDUSTRIES

Horticulture Australia

Quality assurance for improved management of black rot of Brassicas: improved detection and disinfestation in seed; management protocols for seedling and field production

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Media Summary

Black rot and leaf spot diseases of crucifers represent a world-wide problem of economic significance. The causal bacteria, *Xanthomonas campestris* pathovars, are often seed-borne and can go undetected in symptomless but systemically infected seedlings. Water splash resulting from wet and windy conditions favours the dispersal of bacteria and crowding and overhead irrigation, often used in transplant seedling production, significantly increases the likelihood of disease spread. A tolerance of one infected seed per 10 000 clean seeds is considered adequate for direct seeding of brassicas, but a zero tolerance is required for transplant seedling production.

Techniques currently used to identify the presence of *X. campestris* in seed are time-consuming and labour intensive. This project has developed a rapid and sensitive genetic assay to screen batches of brassica seed for the black rot pathogen. The technique can also be used to rapidly confirm infections in plants grown in seedling nurseries and field crops.

The assay targets a molecular marker that distinguishes the pathovars of *X. campestris* causing disease from other bacteria occurring on seed. The assay can reliably detect one contaminated seed in amongst 5 000 clean seeds, and often works at even lower infection levels. The performance of the assay was compared to more traditional methods to detect *X. campestris* and found to be more rapid, reliable and sensitive than the available alternatives. This test will enable growers to be confident that they are using clean planting material, which is an integral part of an IPM strategy aimed at a reducing the incidence of black rot.

Three treatment options to disinfect Brassica seed afflicted by *X. campestris* were evaluated for efficacy against the bacterium, effects on germination, and ease of performance. Treated seeds were examined by bioassay, selective plating and the molecular technique. All treatments reduced the overall microbial load of a sample compared to untreated seeds but none eliminated infection completely. Treatment with copper was the most effective, followed by treatment with bleach and a commercial option.

Despite the prolonged use of copper-containing compounds to control *X. campestris* in *Brassica* field crops, copper resistance does not appear to have emerged in either Australian or foreign isolates of *X. campestris*. Whilst it is fortunate that field isolates of *X. campestris* are sensitive to copper, the potential exists for resistance to develop. Vigilance is necessary to ensure the future efficacy of copper-based control measures against *X. campestris*.

Technical Summary

Black rot of Brassicas can cause severe crop losses when only one seed among 10,000 is infected with the bacterial pathogen, *Xanthomonas campestris*. The culturing techniques employed in the detection of *X. campestris* from infected seed are time-consuming and labour intensive. A molecular assay based on the polymerase chain reaction (PCR) has been developed to provide a rapid and sensitive means for screening Brassica leaves and seed for the black rot pathogen.

Recent reclassification of this species resulted in the number of pathovars of *X. campestris* being amended from over 140 to just six. This includes only those pathovars that cause disease on crucifers; *X. campestris* pvs. *aberrans*, *armoraciae*, *barbarae*, *campestris*, *incanae* and *raphani*. Diseases of Brassica described as black rot and leaf spot have been attributed to pathovars *campestris*, *armoraciae*, *aberrans* and *raphani*. The validity of these distinct pathovars remains uncertain, as suggested by the inclusion of isolates belonging to pathovars *aberrans* and *raphani* within races of *X. campestris* pv. *campestris* (Vicente *et al*., 2001).

The genetic target for a PCR assay would ideally be both necessary and sufficient to cause disease, since this ensures its presence in all pathogenic strains (and absence in non-pathogens). We selected a target gene encoded within the hypersensitive response and pathogenicity (*hrp*) cluster, *hrpF*, whose product is predicted to form part of the plant/bacterial interface (Büttner *et al*., 2002; Rossier *et al*., 2000), and is therefore a potential determinant of host-specificity. PCR primers that detect the *hrpF* locus were designed that effectively differentiate pathovars of *X. campestris* from other *Xanthomonas* sp. and other genera that may occur on Brassica. We did encounter two strains designated *X. campestris* pv. *campestris*, isolated overseas from radish (a crucifer but not a Brassica), that were not detected by this assay; however, their extended maintenance in culture collections may have resulted in the loss of pathogenicity.

An internal control is included to concurrently detect a target unique to the Brassica seed itself (the ITS region), to reduce the incidence of false negative PCR results that may arise from amplification inhibitors within the seed. Despite this, there is the potential for false negatives to occur when the test seed batch carries very few *X. campestris* cells, particularly in combination with large numbers of other microorganisms or high concentrations of PCR inhibitors. The *X. campestris* PCR assay is readily able to detect the target gene directly from infected Brassica leaves and stems, and from extracts of seed washings, with greater speed, selectivity and sensitivity than is possible by existing plating techniques or serological assays.

Three available regimes for the disinfection of Brassica seed infected with *X. campestris* were evaluated. These were treatment with bleach and heat, treatment with copper and heat, and a commercial option. Each was assessed for its efficacy against *X. campestris*, its effect on germination, and ease of performance.

The bioassay is a very useful gauge of the presence of *X. campestris* in seed, providing a true indicator of the persistence of the pathogen following treatment but is labour and time intensive. The PCR

assay permits the sensitive detection of *X. campestris*, but does not offer a means to discriminate between the DNA from living and dead pathogens. Selective plating was also used. The results from the plating of seed washes onto selective media were consistent, with all treatments leading to a reduction in the overall microbial load. *X. campestris* colonies were observed from untreated seed washes and from seed treated with bleach and Incotec. However, the absence of any growth for seed washes from the copper-treated samples suggested that residual copper may be inhibiting microorganisms on the selective media (supported by the observation of a blue tint in the washes of copper treated seed batches). The plating assay results indicated that the most effective treatment was copper, then bleach and the commercial treatment. All options were superior to untreated controls. The bioassay confirmed the presence of black rot in seven of the eight samples.

Despite the prolonged use of copper-containing compounds to control the bacterium in *Brassica* field crops, copper resistance does not appear to have emerged in either Australian or foreign isolates of *X. campestris*. The maximum $CuSO_4$ concentration tolerated by the *X. campestris* strains surveyed, 0.2 mM, is considered to reflect the sensitivity of these isolates to copper. Whilst it is fortunate that *X. campestris* field isolates are sensitive to copper, there is certainly the potential for resistance to develop, particularly via the acquisition of copper resistance plasmids. Vigilance is necessary to ensure the future efficacy of copper-based control measures against *X. campestris*.

CHAPTER 1

Introduction

Pathovars of *Xanthomonas campestris* causing black rot and leaf spot diseases of crucifers represent a world-wide problem of economic significance. The disease is often seed-borne and can go undetected in symptomless but systemically infected seedlings, particularly if climatic conditions are suboptimal for disease expression or when symptoms are obscured by the presence of downy mildew (Williams, 1980). Once bacteria gain entry via hydathodes, stomata or wounds, they may colonise the vascular system, the veins darken; hence the name "black rot" (Figure 1.1). Production of the extracellular polysaccharide, xanthan, restricts water flow and V-shaped chlorotic lesions, characteristic of black rot, develop (Williams, 1980). Water splash resulting from wet and windy conditions favours the dispersal of bacteria from guttation droplets and the rapid spread of disease (Kocks *et al.*, 1999). Crowding and overhead irrigation used in transplant seedling production significantly increases the likelihood of pathogen dissemination, with secondary infections and elevated incidences of disease in the field ensuing (Roberts *et al.*, 1999; Williams, 1980).

A tolerance of one *X. campestris* pv. *campestris* infected seed per 10 000 clean seeds is considered adequate for direct seeding of brassicas, but a zero tolerance is required for transplant seedling production (Schaad *et al.*, 1980b). The initial inoculum carried by an infected seed is a critical factor determining the severity of disease (Roberts *et al.*, 1999), and will vary within and between seed lots. Testing 30 000 seeds, as 3 subsamples of 10 000, has been established as the industry standard for freedom from black rot (Franken *et al.*, 1991).

Fig. 1.1: Characteristic black rot lesion on a cabbage leaf.

Current methods for the detection of *X. campestris* from seed lack sensitivity, are laborious and time consuming, and suspected pathogens are ideally confirmed via pathogenicity testing in plants (Schaad, 1989). This study aimed to develop specific PCR primers that distinguish *X. campestris* from other xanthomonads, and preferably also differentiate the pathovars and races within this species.

If a seed batch is determined to be infected, a number of treatment options are available, but reports of their efficacy and effects on germination have varied (Humayadan *et al*., 1980; Navaratnam *et al*., 1980; Babadoost *et al*., 1996; Kritzman, 1993; Schultz *et al.*, 1986). A comparison of treatment methods for Brassica seed infected with *X. campestris* was undertaken, including treatments described in the literature, and commonly used, and a commercially available (proprietary) alternative. Once an established field crop exhibits symptoms, copper sprays may be applied to minimise the spread of the organism. Since copper resistance is observed among other phytopathogens, including *X. axonopodis* pv. *vesicatoria* (Cooksey, 1990), the copper tolerance of *X. campestris* isolates was assessed, to ascertain the likely effectiveness of this treatment against black rot and to recommend changes in application practices if necessary.

CHAPTER 2

Characterisation of *Xanthomonas campestris* and related species

INTRODUCTION

Members of the genus *Xanthomonas* are Gram-negative rod-shaped bacteria, possessing a single polar flagellum, and usually phytopathogenic (Moffett and Croft, 1983). Traditionally, taxonomy of this genus was determined by the plant host from which the bacterium was isolated (Vauterin *et al.*, 1995). Given the particularly wide host range of *Xanthomonas*, this approach culminated in the type species, *X. campestris,* growing to comprise more than 140 pathovars. Recently however, reclassification of the genus based on DNA-DNA hybridisation studies resulted in the number of pathovars of *X. campestris* being amended to just six (Vauterin *et al.*, 1995). This includes only those pathovars that cause disease on crucifers; *X. campestris* pv. *aberrans*, *X. campestris* pv. *armoraciae*, *X. campestris* pv. *barbarae*, *X. campestris* pv. *campestris*, *X. campestris* pv. *incanae* and *X. campestris* pv. *raphani*.

Diseases of brassicas described as black rot and leaf spot have been attributed to pathovars *campestris*, *armoraciae*, *aberrans* and *raphani* (Zhao *et al*., 2000), which are further divided into races on the basis of interactions with differential *Brassica* cultivars (Vicente *et al.*, 2001). Whilst strains isolated from V-shaped lesions typical of black rot are generally designated pathovar *campestris*, and those from leaf spot as *aberrans*, *armoraciae* or *raphani*, black rot isolates can cause leaf spots when spray-inoculated onto plants for pathogenicity testing (Zhao *et al*., 2000), and plants inoculated with leaf spot isolates have been known to develop lesions indistinguishable from black rot (Moffett *et al.*, 1976). Alvarez *et al.* (1994) observed that there was no molecular or serological basis for the existence of the separate pathovars *armoraciae* and *raphani*, but did find these to be distinct from pathovar *campestris* in pathogenicity tests. The uncertainty surrounding the pathovar distinctions is further demonstrated by the inclusion of the pathotype strains of *aberrans* and *raphani* within race 5 of *X. campestris* pv. *campestris*, based on pathogenicity tests (Vicente *et al.*, 2001).

We utilised a range of methods to characterise representative members of the genus *Xanthomonas*, isolated from a wide variety of plant species. These analyses provided the foundations for the development of a molecular assay to identify those pathovars capable of causing disease in Brassicas, and to distinguish *X. campestris* from other species and genera.

MATERIALS AND METHODS

Bacterial isolates

A collection of over 200 pure cultures of *Xanthomonas* spp. was assembled throughout the course of this project; it included representatives of all *X. campestris* pathovars affecting crucifers (*aberrans, armoraciae, barabare, campestris, incanae*, and *raphani*), in addition to isolates from a wide range of non-cruciferous plants. Bacterial strains used in this study are listed in Table 2.1. Cultures were stored on Protect Beads™ at -80 °C and maintained on nutrient agar (NA; Oxoid) at 25 °C.

Table 2.1: Bacterial isolates used in this study

Species /Strain[1]	Strain name as lodged in other collections[2]	Host plant[3]	Isolation[4] Year	Origin	PCR[5]	ELISA[6]
Xanthomonas albilineans						
X1	DAR 34874	*Saccharum officinarum* (sugar cane)	1980	QLD, Australia	-	nd
Xanthomonas axonopodis* pv. *citri[7]						
X252	DAR 65863	*Citrus grandis* (pommelo)	1991	NT, Australia	-	nd
X253	DAR65869	*Citrus sinensis* (sweet orange)	1984	Torres Strait, Australia	-	nd
Xanthomonas axonopodis* pv. *phaseoli						
X18	DAR 58726	*Phaseolus vulgaris* (French bean)	1987	Australia	-	-
X19	DAR 65944	*Phaseolus vulgaris*	1992	NSW, Australia	-	-
X64[8]	DAR 75943, ICMP 5834, LMG 7455, ATCC 9563, NCPPB 3035	*Phaseolis vulgaris* (green bean)	-		-	-
Xanthomonas axonopodis* pv. *vitians						
X5	PHDS 01/1197	*Lactuca sativa* (lettuce)	2001	NSW, Australia	-	-
X21	PHDS 02/559	*Lactuca sativa* (lettuce)	2002	NSW, Australia	-	-
X43	DAR 31975	*Lactuca sativa* (lettuce)	1978	NSW, Australia	-	+
Xanthomonas axonopodis* pv. *vesicatoria						
X42	DAR 26932	*Datura ferox* L.	1977	NSW, Australia	-	+
Xanthomonas campestris* pv. *aberrans						
X59[8]	DAR 75944, ICMP 4805, LMG 9037, NCPPB 2986; race 5	*Brassica oleracea* (cabbage)	1975	Australia	+	+
X88	DAR 75972, ICMP 4809	*Brassica oleracea* (cauliflower)	1958	Germany	+	-
Xanthomonas campestris* pv. *armoraciae						
X60[8]	DAR 75942, ICMP 7, LMG 535, NCPPB 347	*Iberis* (candytuft)	1954	Tanzania	+	+/-
X89	DAR 75973, ICMP 19, NCPPB 1930)	*Armoracia rustica* (radish)	1939	United States	+	+
Xanthomonas campestris* pv. *barbarae						
X61[8]	DAR 75945, ICMP 438, LMG 547, NCPPB 983	*Barbarea vulgaris* (Upland cress)	1939	United States	+	+
Xanthomonas campestris* pv. *campestris						
X2	DAR 26923	*Brassica oleracea*	1977	NSW, Australia	+	+
X4	DAR 65832	*Brassica oleracea*	1990	TAS, Australia	+	+/-
X7	DAR 30537	*Brassica oleracea* (caulifower)	1978	NSW, Australia	+	-
X8	DAR 30538	*Brassica oleracea* (broccoli)	1978	NSW, Australia	+	+
X9	DAR 65808	*Brassica oleracea* (cauliflower)	1990	NSW, Australia	+	+
X10	DAR 69854	*Brassica tournefortii* (Mediterranean turnip)	1994	NSW, Australia	+	+
X11	DAR 72047	*Brassica oleracea* (cauliflower)	1998	QLD, Australia	+	+
X12	DAR 26914	*Brassica oleracea* (cauliflower)	1975	NSW, Australia	+	+/-
X13	DAR 26921	*Brassica napus* (rape)	1973	NSW, Australia	+	+/-
X14	DAR 26922	*Brassica oleracea* (cabbage)	1973	QLD, Australia	+	+
X33	DAR 72048	*Brassica oleracea* (cauliflower)	1998	QLD, Australia	+	+/-
X34	DAR 75543, ICMP 1683	*Raphanus sativus* (radish)	1963	New Zealand	-	+
X35	DAR 75544, ICMP 3984	*Brassica oleracea* (cabbage)	1974	Cook Islands	+	+
X36	DAR 75545, ICMP 4725	*Brassica oleracea* (Brussels sprout)	1976	New Zealand	+	+
X37	DAR 75546, ICMP 6497	*Brassica oleracea* (cauliflower)	1979	New Zealand	+	-
X46	DAR 75946, ICMP 3	*Brassica napus* (rape)	-	United Kingdom	+	+
X47	DAR 75947, ICMP 6	*Brassica oleracea* (cabbage)	-	Malawi	+	+
X48	DAR 75948, ICMP 8	*Brassica oleracea* (cabbage)	1957	New Zealand	+	+
X49	DAR 75949, ICMP 12	-	1954	India	+	+
X50[8]	DAR 75950, ATCC 33913, NCPPB 528, LMG568, ICMP 13; race 3	*Brassica oleracea* (Brussels sprout)	1957	United Kingdom	+	+
X51	DAR 75951, ICMP 1394	*Brassica oleracea* (cabbage)	1963	United States	+	+
X52	DAR 75952, ICMP 1639	*Brassica juncea* (wild mustard)	-	United States	+	+
X53	DAR 75953, ICMP 9069	*Brassica oleracea* (cabbage)	1982	Hungary	+	+
X54	DAR 75954, ICMP 9071	*Brassica oleracea* (kohl rabi)	1982	Hungary	+	+
X55	DAR 75955, ICMP 10446	*Brassica oleracea* (cabbage)	1947	Ukraine	+	+
X56	DAR 75956, ICMP 10574	*Brassica oleracea* (cabbage)	1989	Yugoslavia	+	+
X57	DAR 75957, ICMP 11929	*Brassica oleracea* (cauliflower)	1975	France	+	-

Table 2.1 (continued)

Species /Strain[1]	Strain name as lodged in other collections[2]	Host plant[3]	Isolation[4] Year	Origin	PCR[5]	ELISA[6]
X58	DAR 75958, ICMP 12188	*Brassica napus* (rape)	1992	Brazil	+	+
X69	DAR 75560	*Brassica oleracea* (cauliflower)	2002	WA, Australia	+	+
X70	DAR 75941	*Brassica oleracea* (cauliflower)	2002	WA, Australia	+	+/-
X71		*Brassica oleracea* (cauliflower)	2002	NSW, Australia	+	+
X71		*Brassica oleracea* (cauliflower)	2002	NSW, Australia	+	-
X73		*Brassica oleracea* (cauliflower)	2002	NSW, Australia	+	+
X74		*Brassica oleracea* (cauliflower)	2002	NSW, Australia	+	+
X75		*Brassica oleracea* (cauliflower)	2002	NSW, Australia	+	+
X76		*Brassica oleracea* (cauliflower)	2002	NSW, Australia	+	+
X77		*Brassica oleracea* (cauliflower)	2002	NSW, Australia	+	+
X78		*Brassica oleracea* (cauliflower)	2002	NSW, Australia	+	-
X79		*Brassica oleracea* (cauliflower)	2002	NSW, Australia	+	+
X80		*Brassica oleracea* (cauliflower)	2002	NSW, Australia	+	+
X81	PHDS 03/87	*Brassica oleracea* (broccoli)	2003	NSW, Australia	+	+
X82	PHDS 03/94	*Brassica oleracea* (cauliflower)	2003	NSW, Australia	+	+
X83	PHDS 03/111	*Brassica oleracea* (broccoli)	2003	NSW, Australia	+	+
X84	PHDS 03/112	*Brassica oleracea* (cauliflower)	2003	NSW, Australia	+	+
X85	PHDS 03/124	*Brassica oleracea* (broccoli)	2003	NSW, Australia	+	+/-
X86	PHDS 03/129	*Brassica oleracea* (broccoli)	2003	NSW, Australia	+	-
X87	PHDS 03/190	*Brassica oleracea* (broccoli)	2003	NSW, Australia	+	+
X90	DAR 75974, ICMP 14	*Brassica oleracea* (cauliflower)	1957	New Zealand	+	-
X91	DAR 75975, ICMP 16	*Raphanus sativus* (radish)	1959	New Zealand	-	+
X92	DAR 75976, ICMP 582	*Brassica rapa* subsp. *sylvestris* (wild turnip)	1962	New Zealand	+	+
X93	DAR 75977, ICMP 2387	*Brassica oleracea* (cauliflower)	1968	New Zealand	+	+
X94	DAR 75978, ICMP 4578	*Brassica oleracea* (cabbage)	1975	Western Samoa	+	+
X95	DAR 75979, ICMP 4784	*Brassica oleracea* (cauliflower)	1976	Tonga	+	-
X96	DAR 75980, ICMP 6498	*Brassica oleracea* (cauliflower)	1979	New Zealand	+	+
X97	DAR 75981, ICMP 7193	*Brassica oleracea* (cabbage)	1980	Fiji	+	+
X98	DAR 75982, ICMP11053	*Brassica oleracea* (cauliflower)	1991	Iran	+	+/-
X101		*Brassica oleracea* (Brussels sprout)	2003	NSW, Australia	+	+
X102		*Brassica oleracea* (cauliflower)	2003	NSW, Australia	+	+
X103		*Brassica oleracea* (cabbage)	2003	NSW, Australia	+	+
	BD63; race 4	*Brassica oleracea* (cabbage)	1997	South Africa	+	+
	BD64; race 1	*Brassica oleracea* (cabbage)	1998	South Africa	+	+
	BD102; race 1	*Brassica oleracea* (cauliflower)	1998	South Africa	+	+
	BD105; race 4	*Brassica oleracea* (cabbage)	1997	South Africa	+	+
	BD116; race 1	*Brassica oleracea* (cabbage)	1998	South Africa	+	+
	BD128; race 4	*Brassica oleracea* (cabbage)	1999	South Africa	+	+
X110	PHDS 03/417	*Brassica oleracea* (cauliflower)	2003	NSW, Australia	+	
X111	PHDS 03/486	*Brassica oleracea* (cauliflower)	2003	WA, Australia	+	nd
X112		*Brassica oleracea* (Brussels sprout)	2003	NSW, Australia	+	nd
X113	PHDS 03/492	*Brassica oleracea* (cauliflower)	2003	WA, Australia	+	nd
X114		*Brassica oleracea* (cauliflower)	2003	NSW, Australia	+	nd
X115	DAR76138	*Brassica oleracea* (cauliflower)	2003	NSW, Australia	+	nd
X116		*Brassica oleracea* (cabbage)	2003	VIC, Australia	+	nd
X117		*Brassica oleracea* (cabbage)	2003	NSW, Australia	+	nd
X118	PHDS 03/659	*Brassica oleracea* (cauliflower)	2003	TAS, Australia	+	nd
X119		*Brassica oleracea* (cauliflower)	2003	VIC, Australia	+	nd
X120		*Brassica oleracea* (cauliflower)	2003	VIC, Australia	+	nd
X122	KX1	*Brassica oleracea*	2000	QLD, Australia	+	nd
X128	MR19	*Brassica oleracea* (cauliflower)	2000	QLD, Australia	+	nd
X131	RBT4	*Brassica oleracea* (cauliflower)	2000	QLD, Australia	+	nd
X156	RBW11	*Brassica oleracea*	2000	QLD, Australia	+	nd
X170	RQ25	*Brassica oleracea* (cauliflower)	2000	QLD, Australia	+	nd
X247		*Brassica oleracea* (cabbage)	2004	QLD, Australia	+	nd

Table 2.1 (continued)

Species /Strain[1]	Strain name as lodged in other collections[2]	Host plant[3]	Isolation[4] Year	Origin	PCR[5]	ELISA[6]
X248		*Brassica oleracea* (cauliflower)	2004	QLD, Australia	+	nd
X249		*Brassica oleracea* (cabbage)	2004	QLD, Australia	+	nd
X250	PHDS 04/205a	*Brassica oleracea* (cabbage)	2004	NSW, Australia	+	nd
X251		*Brassica oleracea* (kohl rabi)	2004	NSW, Australia	+	nd
Xanthomonas campestris* pv. *incanae						
X17	DAR 65877	*Matthiola incana* (stock)	1991	Australia	+	+
X38	DAR 58711	*Matthiola incana* (stock)	1987	NSW, Australia	+	+
X39	DAR 69853	*Cheiranthus cheiri* (wallflower)	1994	NSW, Australia	+	+
X41	DAR 35667	*Matthiola incana* (stock)	1981	NSW, Australia	+	+
X62[8]	DAR 75959, ICMP 574, LMG 7490, ATCC 13462, NCPPB 937	*Matthiola incana (stock)*	1950	United States	+	+
Xanthomonas campestris* pv. *raphani						
X63[8]	DAR 75960, ICMP 1404, LMG 860, NCPPB 1946; race 5	*Raphanus sativus* (radish)	1940	United States	+	+
X99	DAR 75983, ICMP 1641	*Raphanus sativus* (radish)	-	United States	+	+/-
***Xanthomonas campestris* from non-crucifers**						
X31	DAR 69807	*Arfeuillea*	1993	NT, Australia	-	-
X100	PHDS 03/108	*Calathea* sp.	2003	Australia	-	-
X29	DAR 69855	*Cayratia clematidea* (native grape)	1994	NSW, Australia	-	+
X22	DAR 72016	*Citrus paradisi* (grapefruit)	1997	WA, Australia	-	-
X6	DAR 49849	*Daucus carota* (carrot)	1985	NSW, Australia	-	+
X20	PHDS 02/564	*Eriostemon* (waxflower)	2002	Australia	-	-
X3	DAR 61216	*Eriostemon myoporoides* (waxflower)	1988	NSW, Australia	-	-
X25	DAR 54706	*Eriostemon myoporoides* (waxflower)	1986	NSW, Australia	-	-
X32	DAR 73890	*Ficus* (fig)	2000	NSW, Australia	-	-
X16	DAR 61743	*Ficus microcarpa* (fig)	1989	NT, Australia	-	-
X23	DAR 72008	*Lavandula* (lavender)	1996	VIC, Australia	-	+
X24	DAR 72010	*Prunus* (cherry)	1995	VIC, Australia	-	-
X26	DAR 65809	*Prunus persica* (peach)	1990	NSW, Australia	-	-
X27	DAR 65985	*Ranunculus* (buttercup)	1993	NSW, Australia	-	-
X28	DAR 65986	*Ranunculus* (buttercup)	1993	NSW, Australia	-	-
Xanthomonas cucurbitae						
X15	DAR 41331	*Cucurbita pepo* (zucchini)	1983	NSW, Australia	-	-
X65[8]	DAR 75961, ICMP 2299, LMG 690, NCPPB 2597	*Cucurbita maxima* (pumpkin)	1968	New Zealand	-	+
Xanthomonas sesame* pv. *sesame						
X66	DAR 75547	*Sesamum indicum* (sesame)	2002	NT, Australia	-	-
X67[8]	DAR 75558, NCPPB 631	*Sesamum indicum* (sesame)	1958	Sudan	-	-
X68	DAR 75559	*Sesamum indicum* (sesame)	1971	Sudan	-	-
Xanthomonas oryzae* pv. *oryzae						
X44	DAR 61716	*Oryza sativa* (rice)	1987	QLD, Australia	-	-
X45	DAR 61718	*Oryza sativa* (rice)	1987	QLD, Australia	-	-

[1] Species the isolate was received as; in some cases nomenclature has been updated.

[2] Strain designation as held in culture collections: ATCC, American Type Culture Collection, USA; BD, Plant Pathogenic and Plant Protecting Bacteria (PPPPB), Republic of South Africa; DAR, Australian Collection of Plant Pathogenic Bacteria, Australia; ICMP, International Collection of Microorganisms from Plants, New Zealand; LMG, Belgian Co-ordinated Collections of Microorganisms, Belgium; NCPPB, National Collection of Plant Pathogenic Bacteria, UK; PHDS, Plant Health Diagnostic Service, NSW Agriculture, Australia (field isolates collected during this study). Where known, *X. campestris* pv. *campestris* races are given, as designated by Vicente *et al*. (2001).

[3] Host species is given where known, with common name in parentheses.

[4] Year of isolation and state and country of origin are given where known.

[5] +, positive; -, negative in *hrpF* PCR (619 bp).

[6] +, positive; -, negative; +/-, variable or borderline result for *X. campestris* pv. *campestris* ELISA.

[7] DNA extracts only.

[8] Pathotype strain. Additionally, X50 is the *X. campestris* species type strain and the race 3 type strain. X59 is the race 5 type strain.

Extraction of *Xanthomonas* DNA

DNA was extracted from bacterial colonies grown on agar plates using either the Qiagen DNeasy Tissue kit or the Eppendorf Perfect gDNA Blood Mini Isolation Kit. Several colonies were transferred to 1 mL of phosphate-buffered saline (PBS) in a 1.5 mL microfuge tube, and the bacteria were harvested by centrifugation 9000 rpm/ 2 min. The Qiagen DNeasy protocol for animal tissues was then followed, with resuspension of the pellet in 180 µL Buffer ATL. Cells were typically lysed for 1-3 hr and RNase was not added to the extractions. Alternatively, the pellet was resuspended in 1 × PBS and the DNA extracted according to the instructions for saliva.

DNA fingerprinting

AFLP (amplified fragment length polymorphism) analyses were performed using the AFLP Analysis system I AFLP Starter Primer Kit (Gibco BRL). This particular kit is intended for eukaryotic rather than prokaryotic genomes, and contains highly selective primers to reduce the number of potential amplification products from eukaryotic templates. When using this kit for bacteria, the preamplification reaction was performed, but the primer labelling step and selective amplification were omitted, resulting in a suitable number of fragments for analysis.

The protocol described by the manufacturer was followed, with minor modifications and scaled-down reaction sizes. Approximately 120 ng of *Xanthomonas* DNA, extracted using the Qiagen DNeasy kit, was digested in a final volume of 12.5 µL, and adapter ligation was also performed in a final volume of 12.5 µL. The ligations were diluted 1:5 in TE buffer prior to 1 µL being added to the preamplification PCR, to achieve a final volume of 10.2 µL. Cycling conditions were: 1 min at 94 °C, then 35 cycles of 60 sec at 94 °C (denaturation), 1 min at 56 °C (annealing), 1 min at 72 °C (extension), followed by 5 min at 72 °C (extension). Products were separated on a 2.3% agarose gel along with a 100 bp ladder (Pharmacia), stained with Ethidium bromide and visualised by UV transillumination on a GelDoc 2000 (BioRad).

Analysis of Fatty Acid Methyl Esters (FAME)

Identification of bacterial isolates via fatty-acid methyl ester (FAME) analysis was performed by Dorothy Noble and Dr Ric Cother at the Australian Collection of Plant Pathogenic Bacteria, Orange Agricultural Institute, NSW Agriculture.

Analysis by Enzyme Linked Immuno-sorbent Assay (ELISA)

Purified bacterial cultures grown on NA were used in *X. campestris* pv. *campestris*-specific ELISA (Loewe Phytodiagnostics). A match head-sized scraping of culture was resuspended in 1 mL of sample buffer and used directly for ELISA in accordance with the manufacturer's instructions. Each isolate was duplicated within a single microtitre plate. *Pseudomonas syrinage* pv. *tomato* was included as a negative control. A mean absorbance reading of more than double the negative control was considered positive.

Pathogenicity tests

A number of pathogenicity test methods were evaluated, using seedlings and detached leaves of several cultivars and at varying growth stages. A range of inoculation techniques were also employed, including stabbing and crushing of hydathodes, stabbing leaf midveins, cutting leaves, and spray inoculation. Both glasshouse and growth-cabinet conditions were trialled.

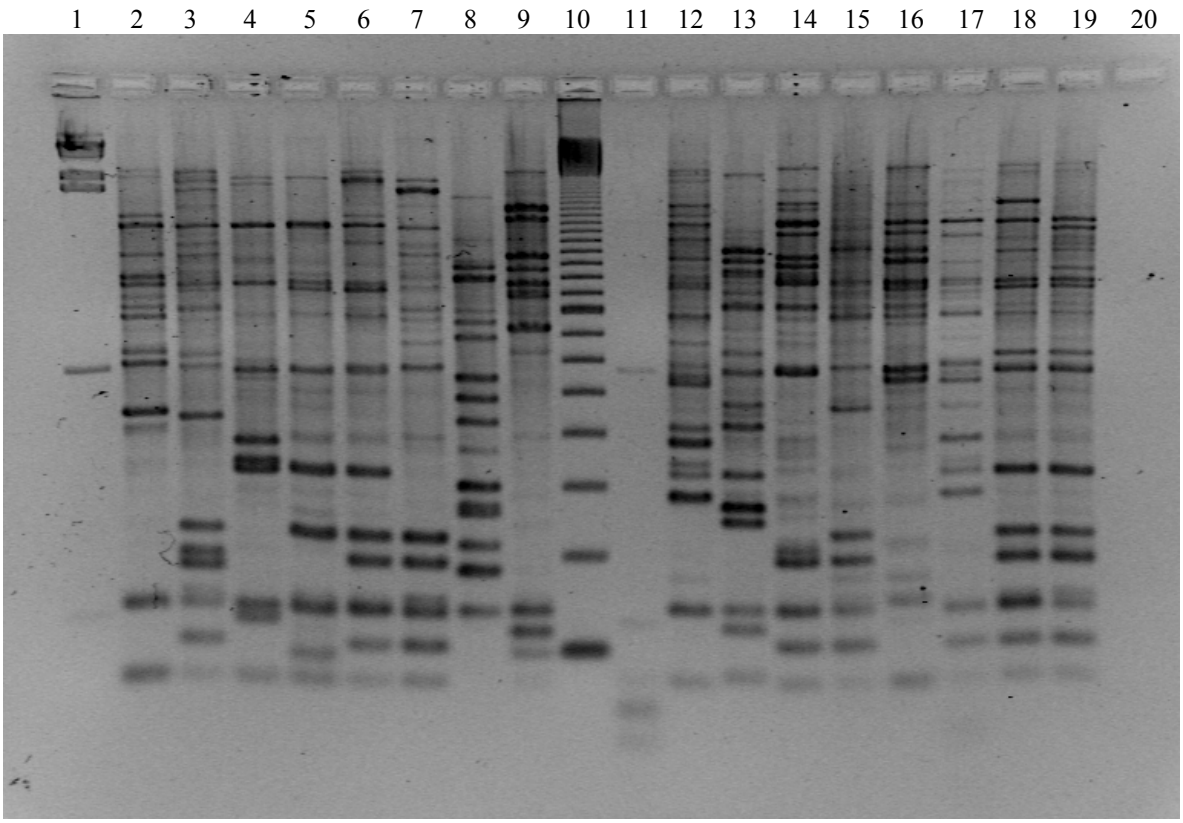
Our preferred method of pathogenicity test was as follows: Healthy *B. oleraceae* var. *capitata* (cabbage cv. Savoy King) seedlings at approximately 4-8 weeks were potted into 10 cm pots and watered well. A suspension of the test isolate was prepared from a 48 hr pure culture on NA, to achieve an OD_{590} of 0.2-0.3 in 0.85% NaCl containing 0.02% Tween 20. Sterile forceps were dipped in the bacterial suspension and then used to crush approximately 5 mm regions at the hydathodes of 2-4 leaves of a cabbage seedling; the forceps were re-dipped in the inoculum between each wounding. A negative control consisted of a plant inoculated with 0.85% NaCl containing 0.02% Tween 20, and a known pathogenic strain was included as a positive control. Pots containing inoculated seedlings were individually transferred to 2 L cylindrical polypropylene screw-top containers, with several small air holes in the lids. Without disturbing the inoculated leaves, water was added to maintain a depth of 1 cm in the bottom of the container throughout the experiment. The seedlings were incubated in a growth cabinet at 26 °C, with 16 hr light each day. The progression of lesions was monitored every 2-3 days for up to 4 weeks, although symptoms in aggressive black rot-inducing strains were generally observed within 1 week.

RESULTS

DNA fingerprinting

AFLP analyses were performed for a representative subset of isolates, comprised of *X. campestris* pathovars, *X. axonopodis* pv. *vesicatoria*, and *Xanthomonas* spp. from *Eriostemon* and *Ranunculus* that appeared closely related to *X. campestris* in other analyses. An example AFLP gel is shown in Figure 2.1. The AFLP profiles of the non-campestris isolates were distinct from those generated from the *X. campestris* strains. Fingerprints unique to each of the *X. campestris* pathovars were not observed; as much variation was apparent within the pathovar *campestris* as was present between this and other pathovars.

Figure 2.1 AFLP fingerprints of *Xanthomonas* spp. separated on a 2.3% agarose gel.



1	λ *Hin*dIII	11	X35 (*X. campestris* pv. *campestris*)
2	X7 (*X. campestris* pv. *campestris*)	12	X38 (*X. campestris* pv. *incanae*)
3	X9 (*X. campestris* pv. *campestris*)	13	X42 (*X. axonopodis* pv. *vesicatoria*)
4	X10 (*X. campestris* pv. *campestris*)	14	X48 (*X. campestris* pv. *campestris*)
5	X11 (*X. campestris* pv. *campestris*)	15	X50 (*X. campestris* pv. *campestris*)
6	X13 (*X. campestris* pv. *campestris*)	16	X61 (*X. campestris* pv. *barbarae*)
7	X14 (*X. campestris* pv. *campestris*)	17	X62 (*X. campestris* pv. *incanae*)
8	*Xanthomonas* sp. from *Erisotemon*	18	X70 (*X. campestris* pv. *campestris*)
9	*Xanthomonas* sp. from *Ranunculus*	19	X72 (*X. campestris* pv. *campestris*)
10	100 bp ladder		

ELISA

One hundred and eight of the strains in our collection were tested by ELISA using an X. *campestris* pv. *campestris*-specific antibody; results are given in Table 2.1. Among the 59 *X. campestris* isolates from Brassicas, 40 (68%) were positive by ELISA, 8 were negative (13%) and 11 (19%) gave a variable, often borderline result (shown as +/- in Table 1). *X. campestris* pathovars *barbarae* and *incanae*, which occur on crucifers but do not affect Brassicas, were also detected by the assay. Positive ELISA reactions were observed for *X. axonopodis* pv. *vesicatoria*, *X. cucurbitae*, *X. axonopodis* pv. *vitians*, and *Xanthomonas* sp. isolated from carrot, cayratia and lavender.

Pathogenicity tests

Several different methods were used to evaluate the pathogenicity of a selection of isolates of Australian origin. The combined results are shown in Table 2.2.

Table 2.2 Summary of pathogenicity tests of *Xanthomonas campestris* isolates on cabbage (cv. Savoy King) seedlings and excised leaves.

(Scoring: 0 No symptoms; 1 Browning or blackening at inoculation site (esp. stab inoculations) but no progression of lesion; 2 Blackening at inoculation site with < 10mm progression along veins; 3 Extensive lesion, progressed >10mm along vein)

Inoculation method / **Isolate (identity *)**	**Spray**	**Dip**	**Spray &/or Stab Hydathode**	**Crush Hydathode**
X69 (Xcc)	3	3	3	-
X72 (Xcc)	3	-	-	-
X14 (Xcc)	3	-	-	-
X39 (Xci)	-	0	-	-
X34 (Xcc)	-	-	1,2	3
X91 (Xcc)	-	-	1	-
X93 (Xcc)	-	-	1	-
X97 (Xcc)	-	-	1	-
X99 (Xcc)	-	-	2	2
X86 (Xcc)	-	-	3	-
X59 (Xcab)	3	-	-	3
X88 (Xcab)	2	-	-	2
X60 (Xcarm)	2	-	-	2
X89 (Xcarm)	2	-	-	2
X104 (Xcc)	-	-	-	3
X105 (Xcc)	-	-	-	3
X114 (Xcc)	-	-	-	3

(*) Identity to pathovar level (Table 2.1). Xcc = *X. campestris* pv. *campestris, Xci = X. campestris* pv. *incanae, Xcab = X. campestris* pv. *aberrans, Xcarm = X. campestris* pv. *armoraciae.*

In general results were the same regardless of the method of inoculation. Crush inoculation of leaves of container-grown seedlings that were then incubated in a cabinet was selected as the simplest and most effective method.

Pathogenicity was observed for most isolates tested. Symptoms did not reflect the nominated pathovar of *X. campestris* – no difference was observed between leaf spot- and black rot-inducing isolates. Not surprisingly, contemporary field isolates (eg X114) showed consistent pathogenicity.

DISCUSSION

The variety of symptoms described for brassicas infected with *X. campestris* may reflect pathovar differences, mode of infection, cultivar-specific host responses, or combinations of these factors. The characteristic symptoms of black rot are blackened veins extending from the leaf margin in a V-shaped lesion, whereas blight is typified by the sudden collapse of large areas of leaf mesophyll, often in the absence of blackened veins; however, blight-affected leaves may later develop symptoms typical of severe black rot (Alvarez *et al*., 1994). Whereas isolates causing typical black rot lesions and blight are routinely assigned to the pathovar *campestris*, designation of leaf spot isolates as either *armoraciae* or *raphani* appears to have been fairly arbitrary (Zhao *et al*., 2000). Necrotic leaf spots (and hydathode necrosis) first emerge as water-soaked spots on the leaf underside, but are soon visible on both leaf surfaces, bordered by a distinct margin; the expanding lesions are eventually limited by the large veins (Moffett *et al*., 1976; Kamoun *et al*., 1992). When pathovars *armoraciae* and *raphani* infect hydathodes, veins in the immediate area may blacken, although invasion of the vascular system does not follow (Kamoun *et al*., 1992). During this study, infected transplant seedlings simultaneously displaying black rot, leaf spot, and stem spot symptoms were observed; furthermore, isolates from leaf spot lesions were found to induce typical black rot symptoms when inoculated onto Brassicas in glasshouse pathogenicity tests. The pathotype strain of *X. campestris* pv. *aberrans* was originally isolated from leaf spot lesions, however, it produced symptoms indistinguishable from black rot in pathogenicity tests at the time (Moffett *et al*., 1976). We observed that inoculation of this *X. campestris* pv. *aberrans* isolate at hydathodes resulted in the formation of tan-coloured lesions consistent with blight (Alvarez *et al*., 1994), which were distinct from the blackened veins caused by other *X. campestris* isolates (data not shown).

The pathotype strains of *aberrans* and *raphani* have been allocated to race 5 of *X. campestris* pv. *campestris*, considered too similar to be distinguished from the pathovar campestris (Vicente *et al*., 2001), whilst the pathovar designations *armoraciae* and *raphani* appear to be interchangeable (Alvarez *et al*., 1994). Ambiguity surrounding the *X. campestris* pathovar and race designations persists; Biolog and FAME identifications tend to be reliable only to the species level (Massomo *et al*., 2003; Zhao *et al*., 2000), and serological, RFLP (Alvarez *et al*., 1994) and genomic fingerprinting analyses (Massomo *et al*., 2003; Zhao *et al*., 2000) have also failed to consistently differentiate the pathovars or races. This lack of evidence for the pathovar distinctions implies that the development of black rot versus leaf spot symptoms may reflect the mode of pathogen entry, whereby infection originating at hydathodes results in the black rot symptoms, and stomatal or wounding infection, such as abrasion (Zhao *et al*., 2000), leads to interveinal leaf spots.

CHAPTER 3

Development of the molecular assay for *Xanthomonas campestris* detection

INTRODUCTION

Whether or not the leaf spot, black rot and blight diseases of Brassicas caused by *X. campestris* are the result of infection by distinct pathovars, all are potentially devastating and merit detection in a seed testing protocol. Ideally, the target of a molecular assay would be both necessary and sufficient to cause disease, since this ensures its presence in all pathogenic strains (and absence in non-pathogens). A number of genetic targets were evaluated for their potential use in the differentiation of those pathovars of *Xanthomonas campestris* that cause disease on Brassicas, from other closely related pathovars, and other species and genera.

MATERIALS AND METHODS

Xanthomonas campestris target selection and primer design

The *hrp* (hypersensitive response and pathogenicity) gene cluster is crucial for the interaction between plant pathogenic bacteria and their hosts, resulting in disease in susceptible plants or the hypersensitive response (HR) in resistant plants (Walton, 1997). The *hrp* gene clusters are largely conserved among phytopathogenic bacteria, where they encode type III secretion systems that deliver pathogenicity factors, elicitors and avirulence proteins to the plant cell (Hueck, 1998; Bonas, 1994). The potential for specific amplification of the *hrp* genes in the detection and identification of *X. axonopodis* pv. *vesicatoria*, affecting tomatoes and capsicums, has previously been investigated (Leite *et al.*, 1994). The *hrp* region genes were examined for their suitability as targets for an *X. campestris* PCR assay, along with other prospective pathogenicity determinants, such as those encoding avirulence factors (*avr, pth*), xanthan gum and extracellular polysaccharide (*gum, rpf*), pigment (*pig, aroE*), metalloproteases, and tannases. Many of these targets were not pursued experimentally, either because the available literature suggested that they were probably dispensable for pathogenicity, or because their nucleotide sequences were too similar to those of other xanthomonads, preventing the design of primers for the specific identification of *X. campestris* pathovars.

Comparison of the available *hrp* sequences, from *X. campestris* pv. *campestris* (da Silva *et al.* 2002; AE008922), *X. axonopodis* pv. *citri* (da Silva *et al.* 2002; AE008923), *X. axonopodis* pv. *vesicatoria* (Huguet and Bonas, 1997; U79116) and *X. oryzae* pv. oryzae (Ochiai *et al.*, 2001; AB045311, AB045312), revealed that the organisation and sizes of the majority of *hrp* genes are highly conserved within the genus, as was predicted from Southern hybridisation analyses described by Bonas *et al.* (1991). Selective amplification of *X. campestris* DNA required a candidate gene that is divergent from its homologues encoded by other *Xanthomonas* species. One of the less conserved genes in this region, *hrpF*, is predicted in *X. axonopodis* pv. *vesicatoria* to encode a membrane-inserted protein that forms part of the translocon mediating delivery of effector proteins across the bacterial-plant interface

(Büttner *et al.*, 2002; Rossier *et al.*, 2000). As such, the HrpF protein may play a role in determining the host specificity of xanthomonads. The *hrpF* gene contains an imperfect direct repeat, two copies of which are seen in the sequences of *X. axonopodis* pv. *citri*, *X. axonopodis* pv. *vesicatoria* (Huguet and Bonas, 1997) and *X. oryzae* pv. *oryzae* (2.42 kb), compared with three copies in *X. campestris* pv. *campestris* (2.76 kb).

On the basis of sequence variation when compared with other available *Xanthomonas* sequences, and their predicted role in pathogenicity, or proximity to pathogenicity determinants a number of loci were evaluated experimentally. These were: *hrpF* and *hrpW*, hypersensitive response and pathogenicity genes; *hrpG*, a regulator of the *hrp* genes that is located approximately 66 kb from the *hrp* region; XCC1218, the gene immediately downstream of *hrpF*, encoding a product of unknown function; XCC2094, a gene encoding a tannase precursor; and *rpoB*, encoding the RNA polymerase subunit B. Whilst unrelated to pathogenicity, *rpoB* has been shown to be useful in the identification and differentiation of other bacterial species, particularly where 16S sequences are highly conserved (Mollett *et al*., 1997; Renesto *et al*., 2001; Lee et al., 2000). Primers designed in the course of this study and used in the assessment of potential targets for the *X. campestris*-specific PCR assay are given in Table 3.1. Forward and reverse primer combinations that were expected to amplify products of up to 1.2 kb were evaluated using PCR.

Table 3.1. Oligonucleotide primers employed in the development of an *X. campestris*-specific PCR assay.

Target gene	Primer name	Primer sequence (5'-3')	Description
hrpF	DLH109	atg tcg ctc aac acg ctt tc	*hrpF* forward primer
	DLH110	cgg agc ttc aga ttg ttt cga c	*hrpF* forward primer
	DLH111	gcg gga aag aat gcc gag ttc	*hrpF* forward primer
	DLH112	gtt ttg cgt gta gcc ctt tgc	*hrpF* reverse primer
	DLH113	gtt gtt caa caa gcc gaa cag	*hrpF* reverse primer
	DLH120	ccg tag cac tta gtg caa tg	*hrpF* forward primer
	DLH124	gca agc tca tcg ccg aca atc	*hrpF* forward primer
	DLH125	gca ttt cca tcg gtc acg att g	*hrpF* reverse primer
	DLH136	cac cgg cta caa gaa gcc cca	*hrpF* forward primer
	DLH137	gga tct tgc tgt ccc act tga g	*hrpF* reverse primer
	DLH148	gat cgg cga ggt tgc cga tgc	*hrpF* forward primer
	DLH149	caa tcg tga ccg atg gaa atg c	*hrpF* forward primer
	DLH150	cat tgc act aag tgc tac gg	*hrpF* reverse primer
XCC1218	DLH114	cat gat cgg act ggg aca gct c	XCC1218 forward primer
	DLH115	cgt aac tcg gtg tcc agt gtc	XCC1218 reverse primer
	DLH121	cgt tcg atg gtg cgt tga tg	XCC1218 reverse primer

XCC2094	DLH116	cta cgg ttt cga gcc gtt cgc	XCC2094 forward primer
	DLH117	gta ttt tgc cgc gac gat gcg	XCC2094 reverse primer
	DLH118	cga ttg aac gga tta cgc gtg	XCC2094 forward primer
	DLH119	cac gac cga ttc gta ata gg	XCC2094 reverse primer
hrpW	DLH122	gat atc agc gtg tcc acc gtt g	*hrpW* reverse primer
	DLH123	cat aag ggc gag gta ttc gac	*hrpW* forward primer
hrpG	DLH126	gca tca cag atc aac gcc agc	*hrpG* forward primer
	DLH127	ctt gta gcc atg cga ata cac g	*hrpG* reverse primer
rpoB	DLH128	gtc atc gac gtg cag gtc ttc	*rpoB* forward primer
	DLH129	ctt cca gca tgc gct gga tc	*rpoB* reverse primer

PCR detection of *Xanthomonas campestris*

For each potential target, the different possible PCR primer combinations were assessed and PCR cycling conditions were optimised with template DNA extracted from a representative subset of the *X. campestris* isolates in our collection. Primer pairs generating a clean product of the expected size were then screened against all isolates in the collection at the time; this comprised a minimum of 78 *Xanthomonas* spp. strains.

PCR was carried out in a final volume of 10 µL, containing 500 nM of each *hrpF* primer, 200 µM dNTPs, 1 × Taq buffer (1.5 mM $MgCl_2$), 1 × TaqMaster PCR enhancer (Eppendorf), 0.5 units MasterTaq DNA polymerase (Eppendorf) and 1 µL template DNA (extracts of pure bacterial cultures, diluted 1:10 in TE buffer). PCR was performed in a Touchdown (Hybaid) thermal cycler with hot lid. Cycling conditions were: 3 min at 95 °C, then 35 cycles of 40 sec at 95 °C (denaturation), 40 sec at 55-63 °C (annealing), 40 sec at 72 °C (extension), followed by 5 min at 72 °C (extension). The annealing temperature was initially selected on the basis of the predicted melting temperatures of the primers and was optimised as necessary. In some instances touchdown PCR was performed, whereby the annealing temperature was reduced by 1°C over the first 6 cycles, to improve the stringency of a reaction.

Amplification products were run on a 1.2-1.5% agarose gel, stained with Ethidium bromide, and visualised by UV transillumination on a GelDoc 2000 (BioRad).

Nucleotide sequencing

A minimum requirement of the assay was that it detected all *X. campestris* isolates that cause disease on Brassicas. All of the targets that met this criterion were also amplified from *X. campestris* pvs. *barbarae* and *incanae*, which do not affect Brassicas, and in some cases, from non-campestris *Xanthomonas* spp. Nucleotide sequencing of such products was undertaken with a view to identifying sequence variation that may be exploited in the differentiation of *X. campestris* from pathovars from one another, and from other species, such as polymorphisms in the recognition sequences of restriction enzymes.

PCR products were purified and concentrated by PEG precipitation prior to quantitation of DNA for sequencing with the PCR primers. Generally, 20 µL of PCR product was precipitated by the addition of an equal volume of PEG solution, containing 26.7% (w/v) PEG_{8000}, 0.6 M NaOAc (pH5.2), and 6.5 mM $MgCl_2$. Samples were incubated for a minimum of 1 hour at 4 °C and DNA pelleted in a microcentrifuge (13 000 rpm/30 min/RT). A drawn out glass pipette was used to remove the supernatant, and the pellet was rinsed once each in 70% EtOH and 100% EtOH, prior to air drying and resuspension in 10-20 µL TE buffer. Approximately 30 ng of product was used in a 45 cycle sequencing reaction with the CEQ 2000 Dye Terminator Cycle Sequencing with Quick Start Kit (Beckman Coulter), in accordance with the manufacturer's instructions. Sequencing products were run on the CEQ 8000 Genetic Analysis System, chromatograms were visually checked with the Chromas software and sequence data was assembled using the BioEdit program.

RESULTS

Development of the *hrpF* molecular assay

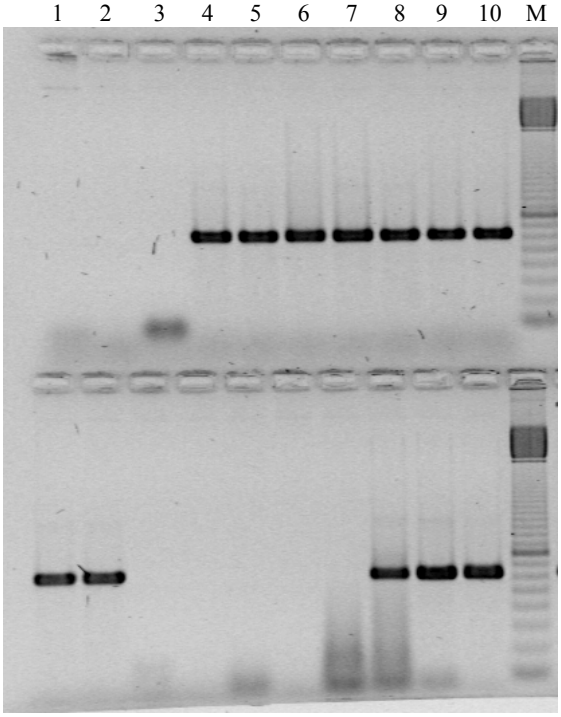
Five primers (DLH109-DLH113) were initially designed to span the repeat sequence at the 5' end of the *hrpF* gene. A collection of over one hundred isolates of *Xanthomonas*, including more than fifty from crucifers, was screened using various combinations of these primers, and the DLH109/DLH112 primer pair was selected for further analysis of the *hrpF* repeat region. These primers generated a 1.4 kb product from *X. campestris* from crucifers, as well as from strains received as *X. campestris* that were isolated from *Eriostemon*, carrot, lavender, ranunculus. The single isolate of *X. axonopodis* pv. *vesicatoria* also produced a 1.4 kb fragment, despite having an expected product of 1 kb based on analysis of the published sequence for this pathovar (Huguet and Bonas, 1997). Products of approximately 1 kb, expected to result from only 2 copies of the direct repeat, were observed for *Xanthomonas* from *Arfeuilla*, *Cayratia* (native grape), *Citrus paradisi* (grapefruit), *Ficus* (fig), *Prunus* (peach, cherry), and *Sesamum indicum* (sesame). Other xanthomonads tested, *X. albilineans*, *X. axonopodis* pv. *vitians*, *X. axonopodis* pv. *cucurbitae* and *X. oryzae* pv. *oryzae*, did not produce a discrete band under the PCR conditions employed. Digestion of the 1.4 kb *hrpF* product with various restriction enzymes (*Hae*III, *Dde*I, *Eco*130I) enabled differentiation of *X. campestris* strains originating on crucifers from other xanthomonads that also produced a 1.4 kb *hrpF* band, but did not distinguish between the pathovars of this species (data not shown). Nucleotide sequencing of representatives of *X. campestris* pv. *campestris* and *X. campestris* pv. *incanae* revealed very few nucleotide differences in this region (data not shown), which could not be detected by restriction digestion of the PCR fragments.

Subsequently, the 3' end of *hrpF*, which showed lower homology to other available sequences, was targeted for the differentiation of *X. campestris* from other species. Primers DLH120 and DLH125 were designed to amplify a 619 bp fragment encompassing the last 580 bp of *hrpF* and 39 bp of downstream intergenic sequence. DLH125 was selected to bind to a non-coding region because this is

more likely to exhibit sequence variation between species and/or pathovars, resulting in a more selective assay. These primers selectively amplified the 619 bp product directly from *X. campestris* DNA extracts. No amplification products were observed from other species of *Xanthomonas*, or from other genera that may occur on Brassicas; *Pseudomonas syringae* pv. *maculicola*, *Pantoea agglomerans* or *Erwinia* sp.

Sequence analysis showed that this region of *hrpF* is too highly conserved among the *X. campestris* pathovars to enable their differentiation from one another (data not shown). Additional primers were synthesised, targeting the middle of the *hrpF* gene, however these were abandoned in favour of the DLH120-DLH125 primer pair, which were particularly robust.

Fig. 2.1. DLH120-125 PCR products run on a 1.3% agarose gel. DNA templates were extracts of pure bacterial cultures.



1	X43 (*X. vitians*)
2	X44 (*X. oryzae* pv. *oryzae*)
3	X45 (*X. oryzae* pv. *oryzae*)
4	X46 (*X. campestris* pv. *campestris*)
5	X47 (*X. campestris* pv. *campestris*)
6	X48 (*X. campestris* pv. *campestris*)
7	X49 (*X. campestris* pv. *campestris*)
8	X50 (*X. campestris* pv. *campestris*)
9	X51 (*X. campestris* pv. *campestris*)
10	X52 (*X. campestris* pv. *campestris*)
M	100 bp ladder
11	X62 (*X. campestris* pv. *incanae*)
12	X63 (*X. campestris* pv. *raphani*)
13	X64 (*X. axonopodis* pv. *phaseoli*)
14	X65 (*X. cucurbitae*)
15	X66 (*X. sesame* pv. *sesame*)
16	X67 (*X. sesame* pv. *sesame*)
17	X68 (*X. sesame* pv. *sesame*)
18	X69 (*X. campestris* pv. *campestris*)
19	X70 (*X. campestris* pv. *campestris*)
20	X71 (*X. campestris* pv. *campestris*)
M	100 bp ladder

Evaluation of other potential molecular targets

The extensive sequence homology shared by the *Xanthomonas* spp. *hrp* regions is less evident toward the *hrp* region boundaries, in the vicinity of genes such as *hrpW*, XCC1218 and *hrpF*, suggesting that an assay designed to target these sequences has the potential to exclusively detect *X. campestris*. XCC1218 is a putative open reading frame (ORF) located immediately downstream of *hrpF* within the *hrp* region. Since the *X. campestris* pv. *campestris hrpF* gene (da Silva *et al.* 2002; AE008922) contained some sequence variation when compared with the equivalent genes in *X. axonopodis* pv. *citri* (da Silva *et al.* 2002; AE008923), *X. axonopodis* pv. *vesicatoria* (Huguet and Bonas, 1997; U79116) and *X. oryzae* pv. *oryzae* (Ochiai *et al.*, 2001; AB045311, AB045312), XCC1218 gene was also investigated as a potential target for a diagnostic assay. Primers DLH114 and DLH115 were designed to non-conserved sequences such that they would amplify an 804 bp XCC1218 product from

X. campestris, however, they did not amplify a product from all *X. campestris* isolates tested. DLH114 was then used in conjunction with a new reverse primer, DLH121, to amplify an expected 509 bp fragment, but a single clean band was not obtained. The XCC1218 reverse primers were also tested with various *hrpF* forward primers but none were successful in detecting all *X. campestris* strains. Little information is available relating to *hrpW*, which was also investigated experimentally as an assay target. The *hrpW* gene lies downstream of XCC1218, and is transcribed in the opposite direction. Primers DLH122 and DLH123 generated a 437 bp product from most *X. campestris* isolates, but a PCR product of this size was also amplified from *Xanthomonas* spp. from non-crucifers such as *Eriostemon*, *Lavandula*, and *Prunus*. Further exploration of this gene was not carried out since *hrpF* was producing more promising results.

The *hrp* region genes are regulated by *hrpX* and *hrpG*, which are located over 60 kb from the *hrp* region itself. Studies in *X. axonopodis* pv. *vesicatoria* have shown that HrpG regulates large numbers of pathogenicity-related genes (*hrp* and avirulence genes), located throughout the genome (Noël *et al*., 2001). The two-component response regulator encoded by *hrpG* requires the transcriptional activator *hrpX* for expression of most genes in the regulon (Noël *et al*., 2001). The *hrpX* gene was considered as a potential target for the *X. campestris* detection assay, but the sequence identity apparent between *X. campestris* pathovars *incanae* and *campestris* suggested that *hrpX* would not provide the level of differentiation sought for the diagnostic assay, and this target was not pursued. In assessing *hrpG* as a PCR target, primers were designed to amplify a 634 bp segment of the gene. Sequence analysis of this fragment was undertaken for 16 strains representing isolates from both leaf spot and black rot lesions, and each of the six *X. campestris* pathovars. Only 10 polymorphisms were identified in this sequence, and none permitted differentiation of the pathovars from one another. This absence of sequence variation, in combination with the difficulties encountered in obtaining a *hrpG* PCR product from all *X. campestris* isolates, dissuaded us from a *hrpG* molecular assay.

X. campestris pv. *campestris*, unlike *X. axonopodis* pv. *citri,* encodes a tannase precursor, XCC2094 (da Silva *et al.* 2002). Tannases enable microorganisms to overcome the inhibitory effect of plant tannins, and thus may be vital for the survival and growth of plant pathogens. Two forward and two reverse primers were designed to target XCC2094; the most promising combination was DLH116-DLH117, which amplified a 609 bp fragment. Subsequent assessment of approximately 80 isolates revealed that only half of the *X. campestris* strains generated the PCR product, furthermore, similar sized products were amplified from several xanthomonads that were not from crucifers, suggesting that this gene is not species-specific.

Although analyses of the 16S rRNA gene and the internal transcribed spacer (ITS) region are widely used in identification of bacteria, the ITS regions of *Xanthomonas* are insufficiently diverged to permit the differentiation of species or pathovars. The universal *rpoB* gene, encoding the RNA polymerase β-subunit, can be a useful alternative since its sequence tends to be more variable (Mollett *et al*., 1997), and *rpoB* has been successfully employed in the differentiation of species of *Borrelia* (Lee et al., 2000) and *Bartonella* (Renesto *et al*., 2001), and serovars of *Salmonella enterica* subsp.

enterica (Kwon *et al*., 2001). Therefore, this gene was explored as the potential target of an *X. campestris*-specific PCR assay. *Xanthomonas*-specific primers were designed for the amplification of a 609 bp variable segment of *rpoB* (the *X. campestris* pv. *campestris* sequence exhibiting approximately 90% identity to *X. axonopodis* pv. *citri*), in the hope that restriction fragment length polymorphisms (RFLPs) could be identified within this region. *rpoB* PCR products were generated from all *X. campestris* isolates tested, and many other xanthomonads. Sequence analysis of this fragment was undertaken for 16 strains representing isolates from both leaf spot and black rot lesions, and each of the six *X. campestris* pathovars. Eleven polymorphisms were identified in this sequence, and although the single *X. campestris* pv. *barabarae* isolate differed from the others at 3 nucleotide positions, differentiation of the pathovars from one another was not achieved beyond this.

DISCUSSION

Our evaluation of a number of genetic loci suggested that the 3' end of the *hrpF* gene represented the most promising target for a PCR assay to detect *Xanthomonas campestris*. The targeted region of the *hrpF* gene appears to occur only in pathogenic strains of *X. campestris*, where it is highly conserved within the species. This absence of sequence divergence precluded *hrpF* from facilitating the differentiation of the *X. campestris* pathovars, prompting further examination of genes located elsewhere on the *X. campestris* chromosome. Approximately 650 bp segments of the *hrp* region regulator, *hrpG*, and the RNA polymerase β-subunit gene, *rpoB*, were sequenced for representative isolates of *X. campestris*, but were found to contain insufficient nucleotide differences for the characterisation of the pathovars (data not shown). Furthermore, none of the sequences examined distinguished the *X. campestris* isolates that caused black rot from those causing leaf spot.

Surprisingly, two strains designated *Xanthomonas campestris* pv. *campestris* and isolated overseas from radish, X34 and X91, were not detected using any possible combinations of *hrpF* primers. Broccoli and cauliflower plants inoculated with either of these strains repeatedly failed to produce infection in a range of pathogenicity tests, despite sequence analyses of the X34 *hrpG* and *rpoB* genes indicating a strong genetic relationship to other black rot-inducing isolates. These strains, and others like them, were found to carry other *hrp* sequences; they had *hrpG* amplification products typical of *X. campestris*, but variable, atypical *hrpW* products. The proximity to one another of *hrpF* and *hrpW* on the *X. campestris* chromosome (approximately 2 kb; da Silva *et al.* 2002) suggests that genetic rearrangements affecting both of these genes may have occurred in the non-pathogenic isolates. In contrast, the *hrp* region regulator, *hrpG*, is situated approximately 66 kb from the genes under its control. These non-pathogenic strains, morphologically indistinguishable from disease-causing *X. campestris* and possessing an incomplete *hrp* region, may represent opportunistic pathogens similar to those described as being isolated alongside pathogenic *X. axonopodis* pv. *vesicatoria* (Stall and Minsavage, 1989). These strains do not induce the hypersensitive reaction and are thought to only contribute to disease development under some circumstances, such as when another organism has initiated a primary infection (Stall and Minsavage, 1989), coincidentally leading to their isolation

from diseased plant material. The finding that *hrpF* negative isolates were also non-pathogenic was consistent with the *hrpF* product being necessary for the development of disease, and with the conclusion of Büttner *et al.* (2002) that *X. axonopodis* pv. *vesicatoria* HrpF is essential for pathogenicity.

CHAPTER 4

Molecular detection of *Xanthomonas campestris* in Brassicas

INTRODUCTION

The black rot bacteria may be carried within the seed, often attached to the funiculus (infection), or simply associated with the surface (infestation; Cook *et al.*, 1952). Both infected and infested seeds are capable of developing disease and acting as a reservoir of infection for surrounding plants. However, recovery of the pathogen from infested seeds is expected to be easier than from infected seeds. Existing seed washing protocols for the detection of *X. campestris* (Schaad, 1989) were adapted for the extraction of DNA and subsequent analysis by PCR. Detection of *X. campestris* directly from bacterial colonies, and from leaf and stem lesions, was also investigated.

The molecular detection method was developed using nucleic acid extracts of pure bacterial cultures, thus the protocol required some refinement for the detection of *X. campestris* from seed. Compounds present in plant extracts, such as tannins, are potential inhibitors of PCR that can give rise to false negative results (Louws *et al.*, 1999). GeneReleaser™ was employed to reduce the effect on the amplification reaction of any inhibitory components present in the plant extracts.

To further ensure the reliability of a negative result, an additional primer set, designed to amplify part of the Brassica genome, were included in a multiplex assay. This target provides an internal positive control for the amplification process, since its presence in the analysed PCR product confirms that the reaction was relatively free of inhibitors (Glick *et al.*, 2002). Failure of the Brassica target to be amplified indicates that a sample probably contains too high a concentration of inhibitory compounds for the amplification reaction to occur.

The PCR-based *X. campestris* detection technique was evaluated against an existing semi-selective plating method (Schaad, 1989) and a commercially available ELISA kit, for sensitivity, selectivity, turnaround time and cost. Detection limits of the PCR assay were established through the testing of seedlots of known infection level, and artificially infected seed batches.

MATERIALS AND METHODS

Template preparation

Extraction of DNA from seed

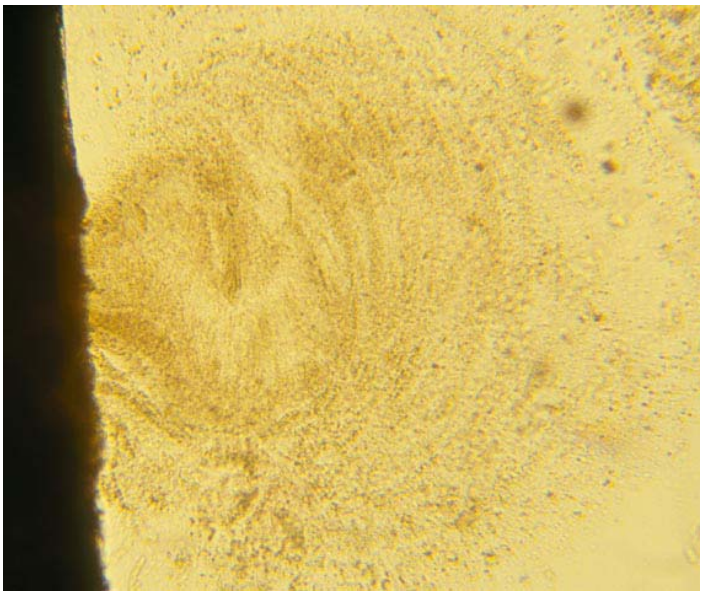
To detect *X. campestris* in seed, washes were performed in a sterile solution of 0.85% saline containing 0.02% Tween 20. Flasks containing 2 mL of wash solution per gram of seed were shaken at 125 rpm/15 °C/3 hr. 1.5 and 10 mL aliquots of the wash liquid were removed for extraction of DNA.

Extraction of DNA was achieved with the DNeasy kit protocol for animal tissues (Qiagen), performed according to the manufacturer's instructions. Starting material consisted of either a toothpick-full of a 48 hr pure culture grown on NA, or an aliquot of the liquid from a seed washing. Samples were washed once in PBS prior to extraction. Eluted DNA was diluted 1:50 and 1:100 in TE buffer for use in PCR, and stored at -20 °C.

Preparation of black rot lesions and bacterial colonies for PCR

Thin sections of leaf or stem lesions suspected to contain *X. campestris* were transferred to a drop of sterile dH_2O and viewed under 100× magnification for the presence of typical bacterial ooze (Schaad *et al.*, 1980b; Figure 4.1). The suspension was aseptically recovered from the slide and used directly in PCR. Suspected *X. campestris* colonies on an agar plate were suspended in 20-50 µL sterile dH_2O and used directly in PCR.

Fig. 3.1 Bacterial ooze from a black rot-affected leaf vein.



Molecular detection

Design of the multiplex assay

To demonstrate the competence of amplification when plant extracts were used as template, these reactions included a second set of primers, DLH138 and DLH139 (Table 4.1), that amplify a segment of the *Brassica* ITS. An alignment of 18S-25S ITS sequences of the Brassicaceae (Yang *et al.*, 1999) was employed in the design of these primers, expected to amplify a 360 bp product from all *Brassica* samples. The ITS primers were designed to be used under the PCR conditions that were previously optimised for the *hrpF* reaction, and were found to be compatible with these conditions in the multiplex PCR. Given that 1500-4300 copies of the rDNA genes are estimated to be present in various *Brassica* species (Bennett and Smith, 1991), and that this smaller target is likely to be preferentially amplified compared with the *hrpF* target, 10 × more of the latter primer pair was used in multiplex PCR.

Table 4.1 Oligonucleotide primers used in a multiplex PCR assay for the detection of *X. campestris*.

Target gene	Primer name	Primer sequence (5'-3')	Description
hrpF	DLH120	ccg tag cac tta gtg caa tg	*hrpF* forward primer
	DLH125	gca ttt cca tcg gtc acg att g	*hrpF* reverse primer
BITS	DLH138	ccc ggc acg aaa agt gtc aag	Brassica ITS forward primer
	DLH139	cct tag ctc gga ttt tgg cc	Brassica ITS reverse primer

Multiplex PCR detection of *Xanthomonas campestris*

For DNA templates derived from seed washings and for PCR directly from infected plant tissue and bacterial colonies, 1 µL of template was added to 6 µL of GeneReleaser™ (BioVentures Inc.), which was overlaid with 20 µL of paraffin oil and microwaved on high for 7 mins, in accordance with the manufacturer's instructions. Tubes were cooled on ice prior to the addition of a master mix. Excluding the oil, these reactions were carried out in a final volume of 16 µL, containing 310 nM of each *hrpF* primer, 31 nM of each *Brassica* ITS primer, 125 µM dNTPs, 1× Taq buffer (1.5 mM $MgCl_2$), 1× TaqMaster PCR enhancer (Eppendorf), and 0.8 units MasterTaq DNA polymerase (Eppendorf).

PCR was performed in a Touchdown (Hybaid) thermal cycler with hot lid. Cycling conditions were: 3 min at 95 °C, 40 cycles of 40 sec at 95 °C, 40 sec at 63 °C (touchdown to 58 °C over the first 6 cycles), 40 sec at 72 °C, followed by 5 min at 72 °C. Amplification products were run on a 1.2-1.5% agarose gel, stained with Ethidium bromide, and visualised by UV transillumination.

Real-time PCR

Real-time PCR is a detection method that offers both a faster turnaround time than conventional PCR, and the potential for a greater degree of sensitivity. In real-time PCR assays, the optimal target size is approximately 70-120 bp; therefore the *X. campestris* multiplex detection assay required some adaptation for real-time PCR. New primers were designed in accordance with Primer3 guidelines, targeting smaller regions within the existing *X. campestris hrpF* and Brassica ITS fragments. The *hrpF* primers were designed to non-conserved sequences (when compared with *hrpF* from *X. axonopodis* pv. *citri*), to ensure their specificity for *X. campestris*, and the Brassica ITS primers designed to sequences that are conserved across the genus.

Primer pairs were initially evaluated in conventional PCR, then in SYBR Green I assays. These were carried out in a final volume of 20 µL, containing 1-1.5 µL of template DNA, 500 nM of each primer, 200 µM dNTPs, 1× Taq buffer (1.5 mM $MgCl_2$), 1× TaqMaster PCR enhancer (Eppendorf), 1 unit MasterTaq DNA polymerase (Eppendorf) and SYBR Green I at a final dilution of 1:20 000-40 000. Alternatively, the Invitrogen Platinum SYBR qPCR Supermix UDG was used in a 20 µL reaction, to which primers and template were added as above. Reactions were performed on a RotorGene (Corbett

Research) with the following cycling conditions: 2 min 30 sec at 95 °C, 30 cycles of 20 sec at 95 °C, 20 sec at 60 °C, 20 sec at 72 °C (acquiring to SYBR channel), 15 sec at 80 °C (acquiring to SYBR channel; this optional step is designed to denature primer dimers), followed by a melt from 72 to 99 °C, increasing by 0.5 °C and holding for 15 sec at each step (acquiring to SYBR channel). Amplification products were also run on a 1.5-2% agarose gel, stained with Ethidium bromide (to stain the size marker), and visualised by UV transillumination.

Once a primer pair had been successfully trialled in a SYBR Green I assay, a dual-labelled assay was performed. These assays incorporate the use of a dual-labelled probe, which binds to one strand of the amplified PCR product, with the 5' end of the probe situated approximately 5 nucleotides from the 3' end of one PCR primer. The additional selectivity afforded by the incorporation of a specific probe permits a greater number of amplification cycles to be performed, thereby maximising the sensitivity of the assay.

Dual-labelled reactions were performed in a final volume of 20 µL, containing 1.5 µL of template DNA, 400 nM of each primer, 200 µM dNTPs, 1× Taq buffer (1.5 mM $MgCl_2$), 1× TaqMaster PCR enhancer (Eppendorf), 1 unit MasterTaq DNA polymerase (Eppendorf), and 200 nM probe. Alternatively, the Invitrogen Platinum Quantitative PCR Supermix-UDG was used in a 20 µL reaction, to which primers and template were added to achieve final concentrations as above. Cycling was performed on a RotorGene (Corbett Research) with the following conditions: 2 min 30 sec at 95 °C, 30-60 cycles of 20 sec at 95 °C, 40 sec at 60 °C (acquiring to the appropriate channel for the probe(s) being used). Occasionally, amplification products were also run on a 1.5-2% agarose gel, stained with Ethidium bromide, and visualised by UV transillumination.

Multiplex dual-labelled reactions were performed in a final volume of 20 µL, containing 1.5 µL of template DNA, 500 nM of each *hrpF* primer, 200 nM of each Brassica ITS primer, 200 µM dNTPs, 1× Taq buffer (1.5 mM $MgCl_2$), 1× TaqMaster PCR enhancer (Eppendorf), and 1 unit MasterTaq DNA polymerase (Eppendorf), 250 nM *hrpF* probe, and 100 nM Brassica ITS probe. As in the conventional multiplex PCR, lower concentrations of the Brassica primers (and probe) were used, to minimise amplification of this more abundant target. The Brassica amplicon was also larger in size, to reduce its amplification efficiency compared with the *hrpF* target.

Table 4.2 Oligonucleotide primers and probes for real-time PCR detection of *X. campestris*.

Target gene	Primer name	Primer sequence (5'-3')	Description
hrpF	DLH151	tag cat caa cag gcc gac t	forward primer for real-time PCR
	DLH152	tat cca gcc gca ctt ctt c	reverse primer for real-time PCR
	DLH153	gta att gat acc gca ctg caa	forward primer for real-time PCR
	DLH154	cac cgc tcc agc cat att	reverse primer for real-time PCR
	P7	atg ccg gcg agt ttc cac g	FAM-labelled probe (on reverse strand) for use with DLH153-154
Brassica ITS	DLH155	caa cgg ata tct cgg ctc tc	forward primer for real-time PCR
	DLH156	ttg cgt tca aag act cga tg	reverse primer for real-time PCR
	P8	cac ggg att ctg caa ttc aca cca	JOE-labelled probe (on reverse strand) for use with DLH155-156

Detection/identification by selective plating

Washings from two naturally infected seed batches were assayed by both PCR and the existing selective plating methods, to compare the sensitivity of these techniques. 10 000 seeds were washed using a method based on that described by Schaad (1989), with the exception that a 15 °C/3 hr wash was performed (as is used for our molecular assay), and the fungicides Benlate and Bravo were omitted from the media. Aliquots were subjected to DNA extraction and PCR, or were plated on the semi-selective media NSCAA (without nitrofurantoin), BSCAA and FS (with and without gentamicin).

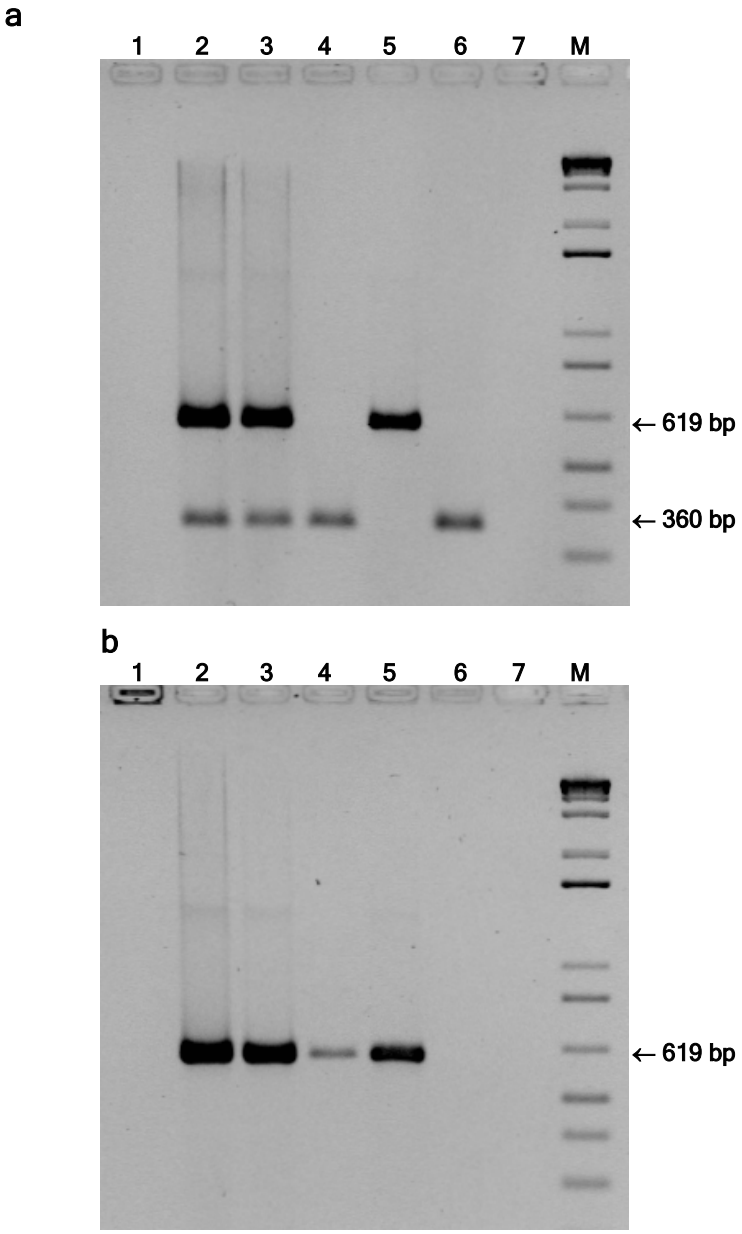
RESULTS

Molecular detection

Detection of *Xanthomonas campestris* from Brassicas using a multiplex PCR assay

This target provides an internal positive control for the amplification process, since its presence in the analysed PCR product confirms that the process was relatively free of inhibitors (Glick *et al.*, 2002). Multiplex PCR also included GeneReleaser™ to minimise the effect of inhibitors and maximise reproducibility, which was not necessary when extracts from pure bacterial colonies were used as PCR templates. The multiplex PCR was successful in detecting *hrpF* and the *Brassica* ITS directly from leaf and stem lesions, and from extracts of infected seed washings (Fig. 4.2).

Figure 2 Multiplex polymerase chain reaction (PCR) products separated on an agarose gel. (a) Multiplex reactions (*Brassica* spp. ITS and *X. campestris hrpF*), (b) *hrpF* reaction; templates are the same in both panels. The 619 bp *hrpF* product is amplified from *X. campestris* and the 360 bp *Brassica* spp. ITS product is amplified from templates derived from plant material. Lane 1, negative control; lane 2, bacterial ooze from a cabbage seedling infected with *X. campestris*; lane 3, extract from heavily infected seed; lane 4, extract from seed carrying a low level infection; lane 5, extract from a pure culture of *X. campestris* pv. *campestris*; lane 6, extract from uninfected seed; lane 7, negative control; M, 1kb+ ladder (Invitrogen, Mount Waverly, Australia).

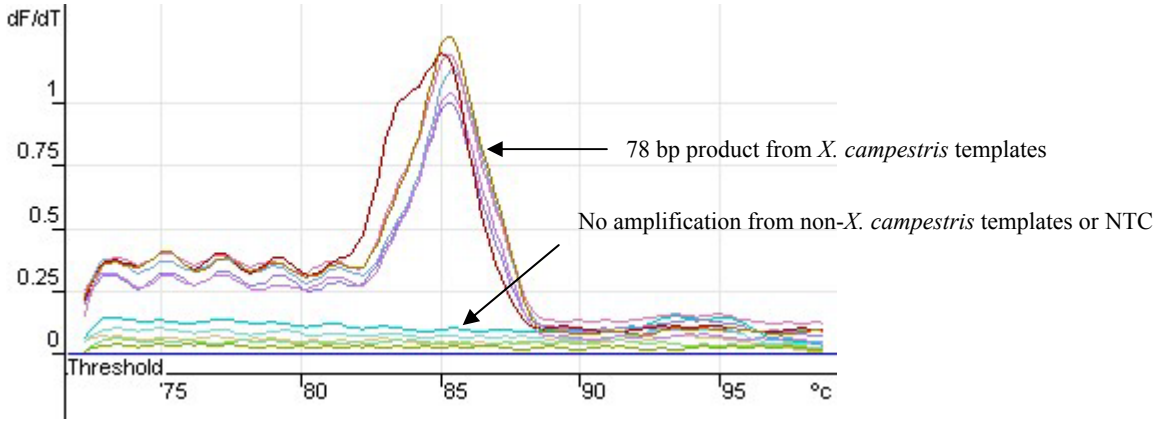


Real-time PCR

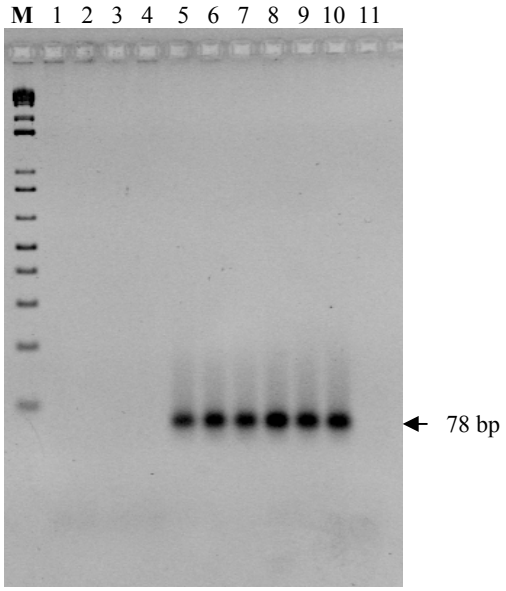
The DLH151-152 and DLH153-154 primer pairs were initially evaluated in SYBR Green I assays, using template DNA from purified *X. campestris*, *Pseudomonas* sp. and *Xanthomonas* sp. from non-crucifers. Primers DLH153-154 produced a single, clean 78 bp band from the *X. campestris* strains (Fig. 3.x), and were subsequently used with a dual-labelled FAM probe (P7) which offers greater sensitivity and specificity. Once this method was successfully implemented, primers DLH155-156, amplifying a 100 bp segment of the Brassica ITS were included in the reaction, along with a specific JOE probe (P8).

Fig. 4.3. Real-time PCR detection of *X. campestris hrpF* and the Brassica ITS region. DNA templates were extracts of pure bacterial cultures.

a) Melt curve for DLH153-154 SYBR Green I real-time PCR.



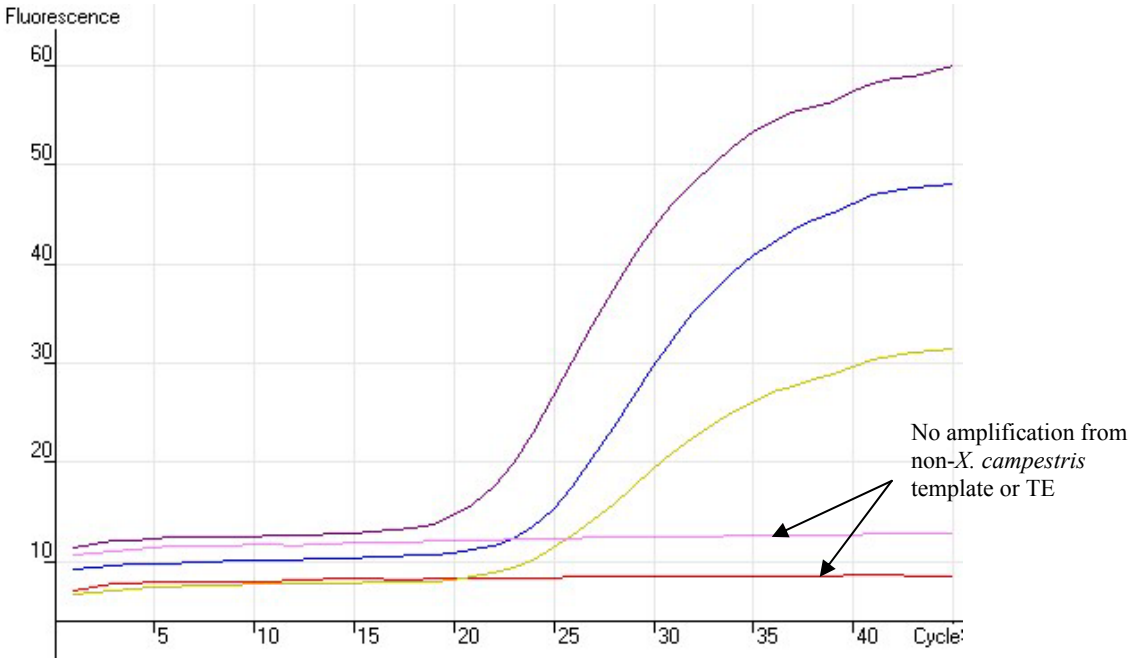
b) Real-time SYBR Green I PCR products run on a 2% agarose gel.



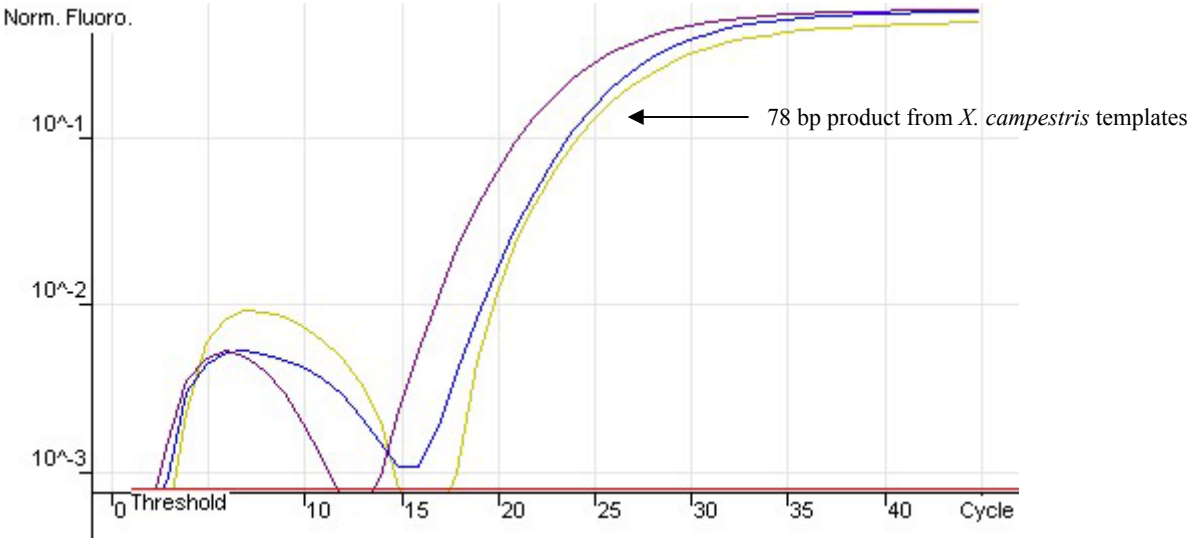
M	1 kb+ ladder
1	X18 (*X. axonopodis* pv. *phaseoli*)
2	X20 (*Xanthomonas* sp. from Eriostemon)
3	X40 (*Pseudomonas* sp.)
4	X66 (*X. sesame* pv. *sesame*)
5	X50 (*X. campestris* pv. *campestris*)
6	X59 (*X. campestris* pv. *aberrans*)
7	X60 (*X. campestris* pv. *armoraciae*)
8	X61 (*X. campestris* pv. *barbarae*)
9	X62 (*X. campestris* pv. *incanae*)
10	X63 (*X. campestris* pv. *raphani*)
11	No template control (NTC)

c) Real-time dual-labelled assay traces (DLH153-154 with the P7 probe). DNA templates were extracts of pure bacterial cultures of *X. campestris* (X59, X60 and X61) and non-campestris *Xanthomonas* sp. (X18), with TE buffer as a negative control. Only the *X. campestris* templates generated the 78 bp *hrpF* amplification product.

Raw data (FAM):

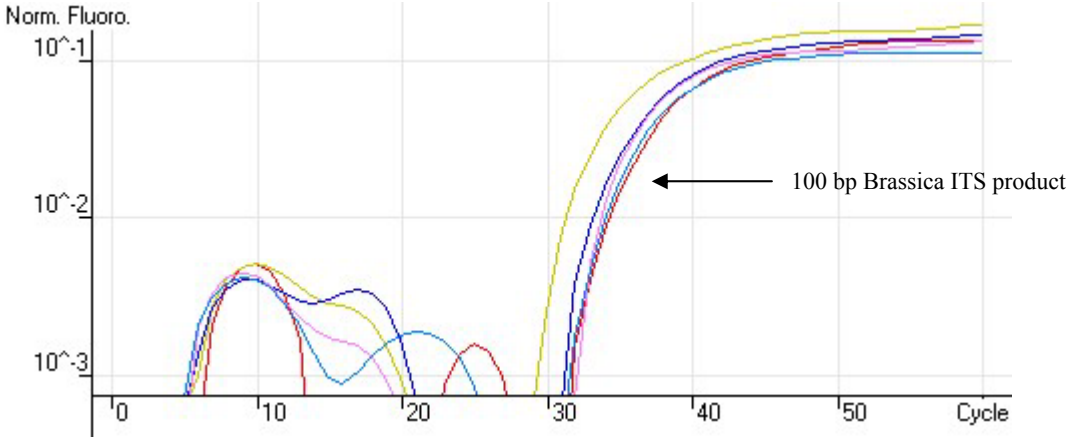


Quantitated data (FAM):

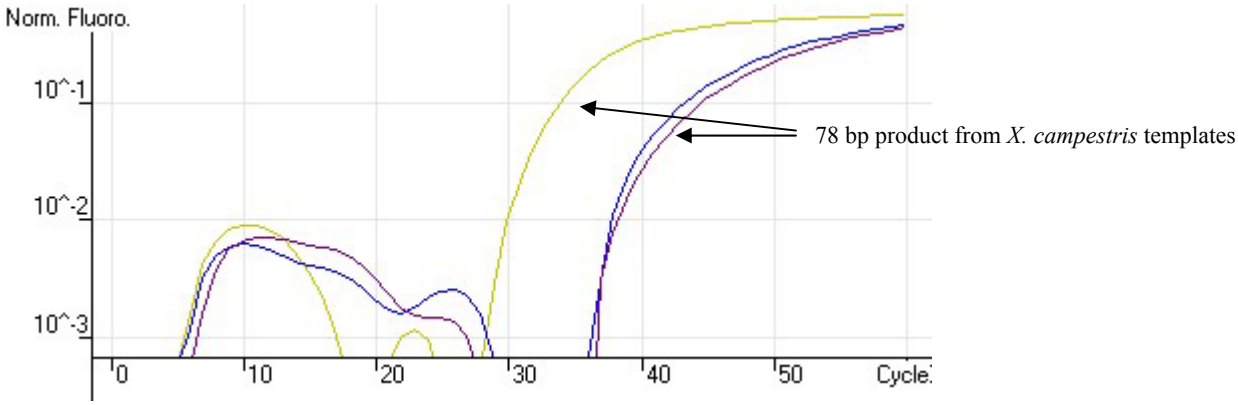


d) Real-time dual-labelled multiplex PCR traces (DLH153-154 with the P7 probe; DLH155-156 with P8 probe). Templates were Brassica seed wash extracts; all samples (except the TE buffer negative control) generated the 100 bp Brassica ITS product (JOE), demonstrating their competence for amplification. The 78 bp *X. campestris hrpF* product (FAM) was amplified from three of the templates, indicating that these contained the pathogen DNA.

Quantitated data (JOE):



Quantitated data (FAM):



Detection/identification on semi-selective agar

Two naturally infected seed batches of *B. oleraceae* var. *capitata* (cabbage) and *B. oleraceae* var. *botrytis* (cauliflower) were employed in a comparison of the PCR assay and the existing selective plating technique (Schaad, 1989). Isolation from semi-selective media was more labour-intensive, time-consuming and ambiguous than the PCR assay. The cabbage seed carried a particularly high microbial load; a variety of other bacteria (and some fungi) frequently overgrew the *X. campestris* colonies, obstructing their identification on the agar plates. The protocol for detection by plating advises that any colonies able to hydrolyse starch on NSCAA medium be further analysed by immunofluorescence and pathogenicity testing on seedlings (Schaad, 1989), which would require a minimum of seven days. The PCR method detected *X. campestris* pv. *campestris* in both seed batches, a result which was obtained within two days.

Selective plating was also used to obtain new isolates from seed, to add to our collection of *X. campestris* from brassicas (Table 2.1). Colonies that hydrolysed starch were generally positive in the *hrpF* PCR; however, several isolates that were morphologically indistinguishable from *X. campestris* on agar were negative for the *hrpF* PCR and did not cause disease when inoculated onto *Brassica* spp. seedlings in pathogenicity tests.

DISCUSSION

The black rot bacteria may be carried within the seed, often attached to the funiculus (infection), or simply associated with the surface (infestation; Cook *et al.*, 1952). Both infected and infested seeds are capable of developing disease and acting as a reservoir of infection for surrounding plants. However, recovery of the pathogen from infested seeds is expected to be easier than from infected seeds, hence the inclusion of an extended wash period that allows the seed to swell and thus may be more likely to release any internalised bacteria. This step did not appear to offer any enrichment of *X. campestris*, but was performed at a temperature and relatively limited time determined to be favourable for their survival (data not shown), while still allowing the seeds to soften. If necessary, washed seeds may be re-dried and subsequently sown without adversely affecting germination (Roberts *et al*., 2002); however, it is likely that any bacteria present in infected seed batches will have spread during the wash step.

Amplification of DNA extracted from aliquots of a seed wash gave a more consistent result when diluted 1:50 and 1:100 in TE buffer, compared with a 1:10 dilution or undiluted DNA, presumably because of the simultaneous dilution of any PCR inhibitors. Similarly, traditional selective plating detection methods generally require the seed wash solution to be diluted 1:100, which is suggested to reduce the inhibitory effects caused by antagonists of *Xanthomonas campestris* that are present in the washes (Randhawa & Schaad, 1984). GeneReleaser™ was employed to reduce the effect on the amplification reaction of any inhibitory components present in the plant extracts, and a marked increase in the quantity and quality of PCR products was observed following the introduction of this

additive. To further ensure the reliability of a negative result, primers that amplify a portion of the *Brassica* ITS were included in a multiplex assay. The presence of this 360 bp product confirms that a reaction was relatively free of PCR inhibitors, permitting the absence of a 619 bp *hrpF* product to be interpreted as a negative result. If the Brassica ITS product is absent, a negative *hrpF* result can not be considered reliable, thus the assay should be repeated. Given that 1500-4300 copies of the rDNA genes are estimated to be present in various *Brassica* species (Bennett and Smith, 1991), and that this smaller target is likely to be preferentially amplified compared with the *hrpF* target, 10× more of the latter primer pair was used in multiplex PCR. The ITS primers were designed to be used under the PCR conditions that were previously optimised for the *hrpF* reaction, and were found to be compatible with these conditions in the multiplex PCR.

CHAPTER 5

Disinfection of Brassica seed infected with *Xanthomonas campestris*

INTRODUCTION

An optimal approach to management of black rot necessitates the use of pathogen-free planting material (Williams, 1980); determination of the black rot status can be achieved via the relatively quick and simple PCR assay. In some circumstances, however, the planting of infected seed will be required, either for breeding purposes or because a desired cultivar is in short supply. There are a number of treatment methods available for seed lots infected with *X. campestris*, but reports vary as to the effectiveness of these methods, and the consequences of each on germination. Treatment methods described in the literature include application of hot water, steam, bleach (NaOCl and CaOCl), cupric acetate, and antibiotics (Minchinton, 1994; Mebalds *et al*., 1997; Humayadan *et al*., 1980; Navaratnam *et al*., 1980; Babadoost *et al*., 1996; Kritzman, 1993; Schultz, 1986; Schaad *et al*., 1980a). Commercially available seed treatment methods also exist, such as the eXccit products offered by the Netherlands-based Incotec company (www.incotec.com). We have assessed several disinfection methods that are routinely used in industry, against both naturally infected and artificially inoculated Brassica seed. The possible implications of antibiotic use in horticulture have been recognised for many years (Schaad *et al*., 1980a), and given the potential for antibiotic resistance to develop in *X. campestris* and other seed-borne bacteria, we elected to exclude antibiotic treatment methods from this study.

Copper is routinely applied to many crops as a fungicide and bactericide, and is essentially the only control mechanism available for outbreaks of black rot; although it is preferable to apply copper-containing sprays to seedlings rather than field crops (Ryan, 1992). Copper resistance has been reported in the bacterial phytopathogens *X. axonopodis* pv. *vesicatoria*, and *Pseudomonas syringae* pv. *tomato* (Cooksey, 1990). Despite decades of copper spray use on Brassicas, the tolerance of *X. campestris* field isolates to copper had only recently been briefly examined (Martin *et al*., 2003). Therefore, the copper sensitivity of a range of the *Xanthomonas* isolates in our collection was determined.

MATERIALS AND METHODS

Disinfection of Brassica seed

Three commonly used disinfection techniques were assessed for efficacy against the black rot pathogen and their impact on germination. Two of these treatment methods, copper acetate and household bleach, are relatively simple to perform, whereas the third, eXccit, is commercially available. Untreated seed batches were included as a control. One naturally infected seed batch and four artificially infected batches were used to evaluate each of the treatment methods.

Sources of infected seed

The artificially infected batches were prepared using a background of *Brassica oleraceae* var. *botrytis* seed (cauliflower cv. Clyde), which had been determined to be *X. campestris*-free in PCR assays, and contained relatively few bacteria or fungi, as ascertained by plating methods. A recent *X. campestris* pv. *campestris* field isolate, X110, was used to inoculate *B. oleraceae* var. *capitata* seeds (cabbage cv. Red Rookie). The pathogenicity of this isolate to Red Rookie and Clyde seedlings was established by inoculation at hydathodes (as described in chapter 2). An OD_{590}=0.3 suspension of X110, containing 2.5×10^8 cfu/mL, was prepared in 0.85% (w/v) NaCl and 0.02% (v/v) Tween 20. Approximately 55 g of Red Rookie seed was submerged in 70 mL of the cell suspension and shaken at 125 rpm for 5 min at RT. The suspension was then removed with a pipette and the seed was spread onto filter paper to dry overnight in a biosafety cabinet. After drying, the seed was stored in a brown paper bag at RT.

The artificially inoculated Red Rookie seeds were added to Clyde seeds to achieve infection levels of 1/10 000, 1/1000, 1/100 and 1/10. Once the seed was germinated, this approach permitted the identification of red cabbage seedlings amongst a background of green cauliflower seedlings, which was required for the bioassays (described below). A naturally infected batch of cabbage seed, with a field infection rate estimated to be between 1/2500 and 1/5000, was also included. Each sample was duplicated.

Fig. 5.1 Black rot-affected *B. oleraceae* var. *capitata* seedlings (cabbage cv. Red Rookie) arising from artificially inoculated seed.

eXccit

The commercially available eXccit treatment, specifically marketed for treatment of *X. campestris* pv. *campestris*-infected *Brassica* seed, was performed by Incotec International, in Holland (a product overview is available at www.seedquest.com/technology/from/Incotec/disinfection/page1.htm). Although a minimum sample size of 167 g (30 000 seeds) is generally required for eXccit treatment, the method was adapted for the 32-40 g sample sizes used in this study. The standard treatment was

applied, although more intensive options are available for particularly heavily infected seed. There is no available information describing the treatment method.

Copper treatment

Seeds were treated with cupric acetate [$(CH_3COO)_2Cu.H_2O$] using a method adapted from those described by Schaad *et al*. (1980a) and Kritzman (1993). A 0.2% (w/v) cupric acetate solution was prepared in pre-warmed reverse osmosis (RO) water containing 0.1% (v/v) glacial acetic acid and 0.02% (v/v) Triton X-100. Ten thousand Brassica seeds (approximately 40 g) were secured in a 15 cm^2 mesh bag, which was immersed in a coffee jar (1 L capacity) containing 400 mL of the cupric acetate solution. The seeds were incubated in a waterbath for 20 min with gentle shaking. The temperature within the jar was monitored and maintained at 40 °C ± 0.5 °C for the duration of the treatment. The cupric acetate solution was tipped off and the jar twice filled with tap water to rinse the seeds. The seed bag was shaken to remove excess liquid, gently squeezed in blotting and spread on blotting paper to dry overnight in a biosafety cabinet (with several changes of paper). Once dried, the seed was stored in a brown paper bag at RT.

Bleach

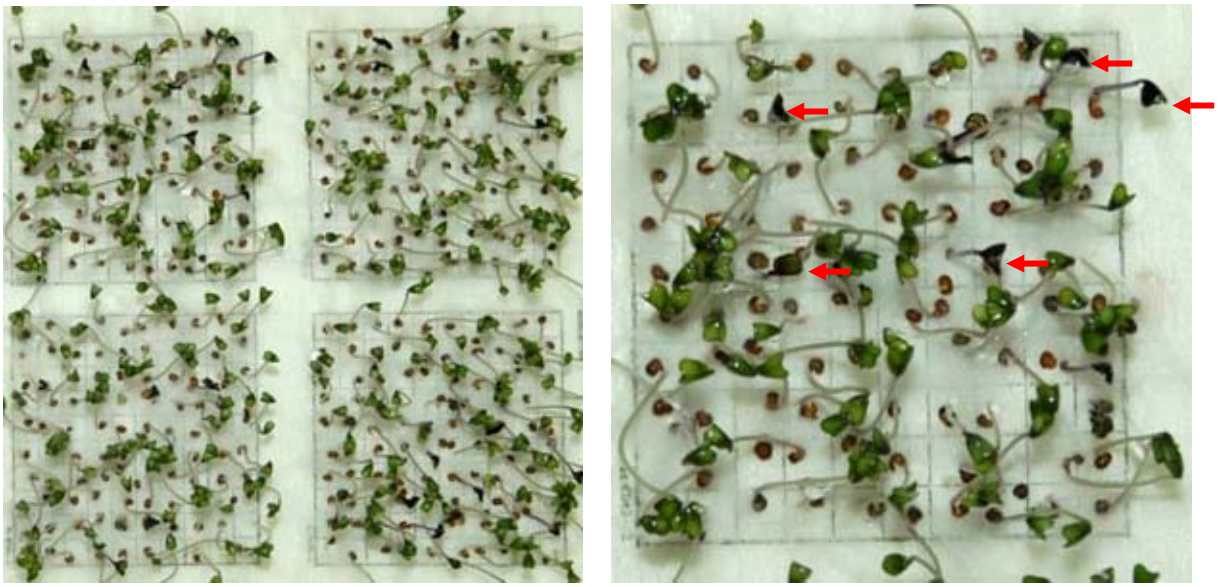
Bleach treatment was performed in 1 L glass jars, in mesh bags as described above. A previously unopened bottle of household bleach (BiLo brand; 4% active chlorine) was used to prepare the 0.01% (v/v) treatment solution in pre-warmed RO water. In a gently shaking waterbath, 40 g seed batches were treated in 400 mL of bleach solution for 20 min at 50 °C ± 0.5 °C. The bleach solution was tipped off and the mesh bag suspended in a 2 L measuring cylinder filled with tap water. A 10 min rinse under running water was achieved by adding water to the bottom of the cylinder. The seed was dried and stored as described for the cupric acetate treatment.

Germination assays

The effect of seed treatments on germination was assessed via seed germination assays, which were performed as described in the International Rules for Seed Testing (1999). For brassicas, this essentially required 4×100 seeds of each sample to be germinated on top of blotting paper at 20-30 °C, and scored at 5 and 10 days. The assays were performed on the bench in 18×28 cm trays with clear plastic covers. Blotting paper was kept moist via the addition of sterile dH_20 (pH 6.5-7.0). Seedlings were considered to have germinated normally if the following structures were developed or clearly developing: root system, shoot axis, cotyledons. Seed was scored as abnormal if germination had commenced but the seedling was significantly malformed. Ungerminated seed was that which had either imbibed water but not actually germinated, or seed that remained hard.

Fig 5.2 Germination assay. A: Seeds from a 1/10 infection rate sample on a 4×100 grid at 5 days. B: Red arrows indicate the positions of several Red Rookie red cabbage seedlings on one grid.

A **B**



Bioassays

Bioassays were performed to determine the efficacy of each seed treatment against the black rot bacterium. One thousand seeds of each of the highest infection rate samples (1/10) were sown in 240 cell seedling trays (2 seeds per cell), in seed raising mix overlaid with vermiculite. Seedlings were germinated and raised in a glasshouse with an average daytime temperature of 26 °C, during April 2004, without supplemental lighting. Samples for each treatment were housed on separate benches to avoid cross-contamination and water was delivered via overhead misters (3 times per day for 5 min). Although scoring was intended to be done 28 days after sowing, the appearance of downy mildew on some seedlings prompted us to instead score at 26 days. To confirm that symptomatic leaves were carrying *X. campestris*, and asymptomatic leaves were pathogen-free, a selection of leaves was examined in the laboratory. These were checked microscopically for the presence of bacterial ooze (as described in chapter 4) and evidence of downy mildew infection. The contents of the slides were aseptically recovered, plated on King's Medium B, and assayed by PCR to detect *X. campestris*.

Fig. 5.3 Glasshouse setup for bioassays

Seed wash assays

Subsamples of each seed batch were washed in sterile 0.85% saline containing 0.02% Tween 20. The washes were performed in sterile 100 mL specimen jars with shaking at 125 rpm/15 °C/3 hr (the jars were incubated on their sides to maximise agitation). 8 000 seeds of each 1/10, 1/100, 1/1 000 and naturally infected sample were washed in 64 mL of buffer. Since the 1/10 000 samples carried a very low infection rate, all available seeds were washed (approximately 9 600; 400 were used in the germination assay) in 80 mL of buffer.

On completion of the wash step, 10^{-1} dilutions of the wash solutions were prepared in sterile 0.85% saline. Aliquots of the undiluted and 10^{-1} samples (100 µL) were spread onto NSCAA media containing nitrofurantoin and the plates were incubated at 25 °C for 72 hr. The plates were then stored at 4 °C for 24 hr prior to scoring, and selected colonies were assayed by PCR to confirm their presumptive identification as *X. campestris* or other.

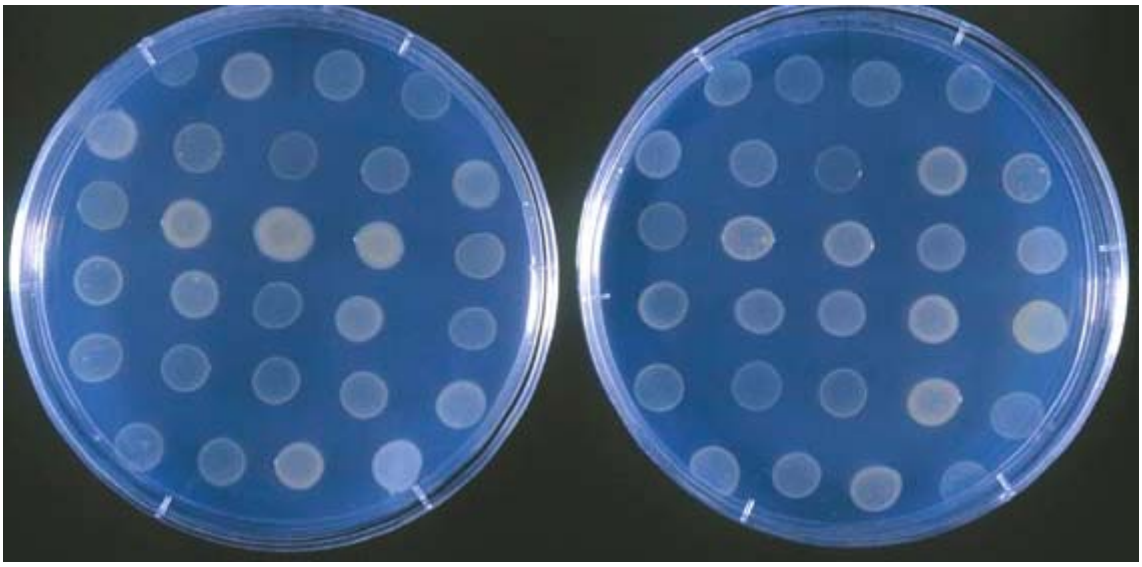
Aliquots of each seed wash (1.5 mL) were also subjected to DNA extraction using the Eppendorf Perfect gDNA Blood Mini Isolation Kit (this kit does not yield as much DNA as the Qiagen DNeasy kit, but the extractions are quicker and simpler to perform in bulk). Samples were pelleted in a microcentrifuge (10 000 rpm/3 min) and washed once in PBS prior to extraction as described in the manufacturer's instructions. Eluted DNA was stored at 4 °C and diluted 1:50 in TE buffer for use in PCR as described in chapter 4.

Assays to evaluate resistance to copper

Copper resistance assays were performed using a method adapted from those described for *Pseudomonas syringae* (Scheck *et al*., 1996; Andersen *et al.*, 1991; Tesoriero *et al*., 1997). Casitone-yeast extract-glycerol agar (CYEG) was prepared and the pH adjusted with NaOH to compensate for the drop in pH that occured on addition of copper sulfate, giving a final pH of 6.5-7. A 50 mg/mL (approximately 200 mM) stock solution of $CuSO_4.5H_2O$, prepared in sdH_2O and filter-sterilised, was added to the cooled agar prior to pouring. In preliminary experiments, $CuSO_4$ was added to achieve eight final concentrations ranging from 0.1 to 2.0 mM, however, once it was apparent that *X. campestris* was relatively copper sensitive (compared to *Pseudomonas syringae*), concentrations were reduced and the copper resistance assays were completed on CYEG containing 0, 0.1, 0.2, 0.3, 0.4, and 0.5 mM $CuSO_4$.

Suspensions of inocula were prepared in 0.85% NaCl containing 0.02% Tween 20, from 48hr cultures on NA, to an OD_{590} of 0.22-0.28. A total of 107 *X. campestris* strains (X2-39, X41-109; Table 2.1) were assessed for copper resistance, by spotting 10 µL onto the copper-amended media. Copper-resistant *Pseudomonas syringae* pv. *tomato* PT23 was included as a positive control. Growth on the plates was scored following 48 hr incubation at 25 °C (Fig. 5.4). The copper tolerance of each isolate was determined in triplicate and the results of the three independent experiments were averaged.

Fig. 5.4. *X. campestris* isolates on CYEG agar (copper-free control), following 48 hr incubation.



RESULTS

Disinfection of Brassica seed

Germination assays

The *B. oleraceae* var. *botrytis* (cauliflower cv. Clyde) seed had previously been observed to have a germination rate of 96%, and *B. oleraceae* var. *capitata* (cabbage cv. Red Rookie) seed had a germination rate of 97%. Germination rates were established for the artificially infected samples carrying the highest (1/10) and lowest (1/10 000) rates of inoculated Red Rookie seed, in a background of clean Clyde seed.

The results of the germination assay are presented in Table 5.1. Each of the treatments reduced the germination frequency to some extent compared with the untreated seed (which had an average germination rate of 97%), however the effect was minimal. The bleach and Incotec treatments both reduced germination to 94%, whilst the cupric acetate-treated seeds had an average germination rate of only 89%.

Table 5.1 Results of germination assays

Seed treatment and starting infection rate	Germination (%)		Germinated + normal	Average normal germination (%)
	5 days	10 days	10 days	
Untreated				
1/10 000 (A, B)	98, 97	98, 98	97, 97	
1/10 (A, B)	97, 98	98, 98	97, 98	97
Bleach treatment				

Seed treatment and starting infection rate	Germination (%)		Germinated + normal	Average normal germination (%)
	5 days	10 days	10 days	
Bleach treatment				
1/10 000 (A, B)	93, 94	96, 95	94, 95	
1/10 (A, B)	90, 90	92, 94	92, 94	94
Copper treatment				
1/10 000 (A, B)	90, 86	95, 90	93, 87	
1/10 (A, B)	86, 83	88, 91	85, 89	89
eXccit treatment				
1/10 000 (A, B)	95, 92	97, 95	94, 93	
1/10 (A, B)	93, 94	94, 96	93, 95	94

Bioassays

Bioassays were performed to ascertain the success of each treatment against the black rot pathogen. Only those samples containing higher initial infection rates were included in the bioassays, since a subsample of these would contain sufficient inoculated Red Rookie seedlings to observe any changes in the infection level post-treatment. For each treatment, 1000 seeds were germinated and assessed for the presence of black rot symptoms after 26 days (Table 5.2).

Fig. 5.5. Symptoms of infection with *X. campestris* Left: Affected Red Rookie seedlings amongst affected *B. oleraceae* var. *botrytis* (cauliflower cv. Clyde) (infection spread) Right: symptoms on infected *B. oleraceae* var. *botrytis* (cauliflower cv. Clyde).

Table 5.2 Bioassay results as scored 26 days after sowing.

Seed treatment and starting infection rate	Surviving Red Rookie seedlings	Red Rookie seedlings with symptoms	Patches of *X. campestris* infection (no. of seedling tray cells affected)	Percentage of seedlings affected
Untreated				
1/10 (A)	58	17	1 × 40	4.6
			1 × 6	
1/10 (B)	97	20	1 × 30	13.5
			1 × 15	
			1 × 90	
Bleach treatment				
1/10 (A)	56	23	1 × 120	13.6
1/10 (B)	50	2	1 × 12	1.6
			1 × 4	
Copper treatment				
1/10 (A)	10	0	0	0
1/10 (B)	19	2	1 × 20	2.0
eXccit treatment				
1/10 (A)	76	18	1 × 30	8.5
			1 × 25	
			2 × 15	
1/10 (B)	67	9	1 × 40	4.0

To confirm the assessment of the seedlings in the bioassays, leaves were harvested from asymptomatic seedlings and those exhibiting a range of symptoms, of both Red Rookie and Clyde. These were examined for bacterial ooze and the majority were cultured on King's medium B and assayed by PCR for the presence of *X. campestris*. A variety of symptoms were observed, including typical V-shaped chlorosis accompanied by blackening of veins and water-soaked leaf spots that originated on the leaf underside. A strong correlation was observed between the presence of water-soaked leaf spots or blackened veins, the observation of bacterial ooze from leaf sections, subsequent isolation of *X. campestris* on King's medium B, and a positive result in the PCR assay (Table 5.x).

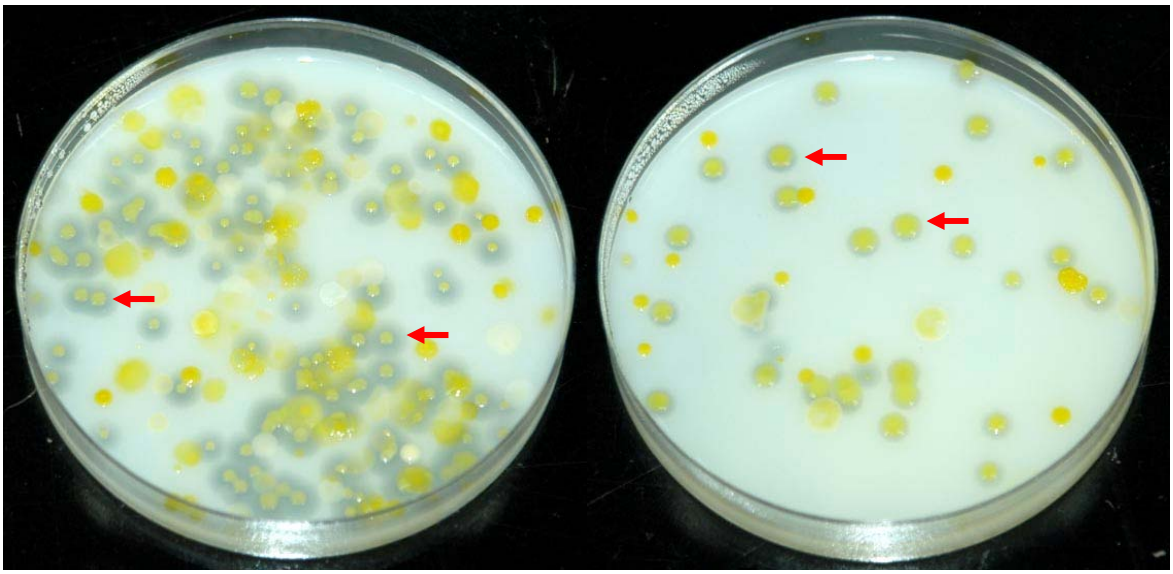
All patches of disease contained a black rot-affected Red Rookie seedling in the centre. Generally, the Clyde seedlings exhibited leaf spot symptoms.

Seed wash assays

Subsamples of the treated seeds were washed and assayed for the presence of *X. campestris* by plating on selective media. The combined colony counts for the replicates of each seed batch are shown in Table 5.3. Up to eight colony types (including fungi) were observed on the plates for untreated seed. *X. campestris* colonies appeared as pale (milky) yellow, shiny, mucoid colonies, surrounded by a clear zone of starch hydrolysis on NSCAA media (Fig. 5.6). Several other starch-hydrolysing yellow colony types were observed, however these were very dry in texture when touched with a loop, and were negative when subsequently assayed by PCR (Table 5.6).

The highest colony counts of *X. campestris* and other organisms were observed on the plates from untreated seed. Determination of the exact number of colonies on several of these plates was not achieved due the variety of colony types, the high number of colonies present and the opacity of the media; in such cases estimates were made and the *X. campestris*-like colonies were counted where possible.

Fig. 5.6 NSCAA plates from untreated seed washes. Selected *X. campestris* colonies are indicated by red arrows.



Extracts of the seed washes were used as templates for both the *hrpF* and multiplex (*Brassica* spp. ITS and *hrpF*) PCR assays. The bleach treatment appeared to have a significant inhibitory effect on the amplification process; several extracts from bleach-treated seeds failed to amplify the *Brassica* ITS, and all products generated from these extracts were less abundant than those from other samples (data not shown). All of the extracts from treated seed exhibited a reduced capacity for amplification, compared with extracts of untreated seed (bleach<copper<Incotec<untreated). The difficulty in detecting very low rates of infection is demonstrated by our success in amplifying *hrpF* from only one replicate of the 1/10 000 infection rate sample. Detection of *X. campestris* by PCR was much more sensitive than detection on selective media.

Table 5.3 Results of PCR and selective plating assays for treated seed.

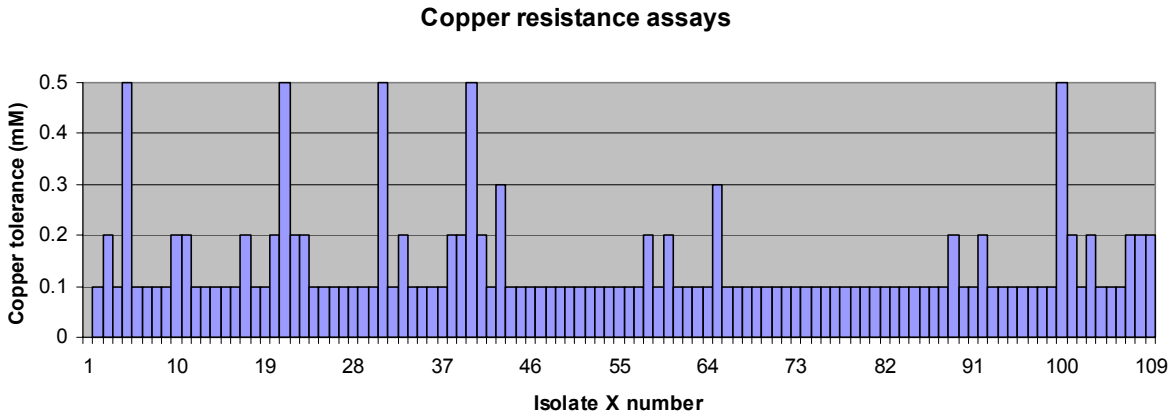
Sample	Seed wash PCR (*hrpF*)	Seed wash PCR (BITS)	NSCAA plates	Total colony count	Starch hydrolysing colony count	NSCAA colony PCR result
Untreated						
1/10 (A)	+	+	undiluted	>500	-	+
			10^{-1}	105	**63**	
1/10 (B)	+	+	undiluted	>500	-	
			10^{-1}	approx. 400	**52**	
1/100 (A)	+	+	undiluted	>500	-	-
			10^{-1}	137	14 (bright yellow)	
1/100(B)	+	+	undiluted	approx. 300	-	+
			10^{-1}	42	28 (orange; only **2** are Xc-like)	
1/1000 (A)	+	+	undiluted	211	16	-
			10^{-1}	19	3	
1/1000 (B)	+	+	undiluted	approx. 100	21 (orange)	
			10^{-1}	15	0	
1/10 000 (A)	+	+	undiluted	approx. 100	-	
			10^{-1}	34	2	
1/10 000 (B)	-	+	undiluted	approx. 300	white contam.	
			10^{-1}	1	1	
Natural (A)	+	+	undiluted	>500	-	+
			10^{-1}	approx. 500	**231**	
Natural (B)	+	+	undiluted	approx. 500	-	-
			10^{-1}	approx. 230	60	
Bleach treatment						
1/10 (A)	+	+	undiluted	2	**2**	+
			10^{-1}	0	0	
1/10 (B)	+	+	undiluted	0	0	
			10^{-1}	0	0	
1/100 (A)	-	+	undiluted	0	0	
			10^{-1}	0	0	
1/100(B)	+	+	undiluted	1	0	
			10^{-1}	0	0	
1/1000 (A)	-	+	undiluted	0	0	
			10^{-1}	0	0	
1/1000 (B)	-	+	undiluted	0	0	
			10^{-1}	0	0	
1/10 000 (A)	-	-	undiluted	0	0	
			10^{-1}	0	0	
1/10 000 (B)	-	-	undiluted	0	0	
			10^{-1}	0	0	
Natural (A)	+	+	undiluted	0	0	
			10^{-1}	0	0	
Natural (B)	+	+	undiluted	1	0	-
			10^{-1}	0	0	
Copper treatment						
1/10 (A)	+	+	undiluted	0	0	
			10^{-1}	0	0	
1/10 (B)	+	+	undiluted	0	0	
			10^{-1}	0	0	
1/100 (A)	-	+	undiluted	0	0	
			10^{-1}	0	0	
1/100(B)	+	+	undiluted	0	0	
			10^{-1}	0	0	
1/1000 (A)	-	+	undiluted	0	0	

Sample	Seed wash PCR (*hrpF*)	Seed wash PCR (BITS)	NSCAA plates	Total colony count	Starch hydrolysing colony count	NSCAA colony PCR result
			10^{-1}	0	0	
1/1000 (B)	-	+	undiluted	0	0	
			10^{-1}	0	0	
1/10 000 (A)	-	+	undiluted	0	0	
			10^{-1}	0	0	
1/10 000 (B)	-	+	undiluted	0	0	
			10^{-1}	0	0	
Natural (A)	+	+	undiluted	0	0	
			10^{-1}	0	0	
Natural (B)	+	+	undiluted	0	0	
			10^{-1}	0	0	
eXccit treatment						
1/10 (A)	+	+	undiluted	29	**29**	+
			10^{-1}	3	**3**	
1/10 (B)	+	+	undiluted	9	**9**	+
			10^{-1}	1	**1**	
1/100 (A)	-	+	undiluted	1	0	
			10^{-1}	22	0	
1/100(B)	+	+	undiluted	20	0	-
			10^{-1}	3	0	
1/1000 (A)	-	+	undiluted	0	0	
			10^{-1}	0	0	
1/1000 (B)	-	+	undiluted	0	0	
			10^{-1}	0	0	
1/10 000 (A)	-	+	undiluted	0	0	
			10^{-1}	0	0	
1/10 000 (B)	-	+	undiluted	0	0	
			10^{-1}	0	0	
Natural (A)	+	+	undiluted	0	0	
			10^{-1}	0	0	
Natural (B)	+	+	undiluted	0	0	
			10^{-1}	0	0	

Copper resistance assays

The results of the copper resistance assays are given in Fig. 5.7. All of the *X. campestris* isolates tested were sensitive to copper; growth was inhibited on CYEG containing 0.1 or 0.2 mM $CuSO_4$. *Xanthomonas* spp. isolated from *Afeuillea* (X31) and *Calathea* (X100) were tolerant to 0.5 mM $CuSO_4$, and the three strains originating from lettuce (X5, X21 and X43) exhibited resistance levels ranging from 0.3 to >0.5 mM. The *X. cucurbitae* type strain (X65) was also tolerant to 0.5 mM $CuSO_4$.

Figure 5.7 Copper tolerance of *Xanthomonas* isolates. The maximum $CuSO_4$ concentration in CYEG agar at which bacterial growth was observed is shown.



DISCUSSION

Three treatment options available for Brassica seed infected with *X. campestris* were evaluated for efficacy against *X. campestris*, effects on germination, and ease of performance. Thorough examination of the treated seeds by bioassay, selective plating and PCR techniques also provided insights into the usefulness of testing for *X. campestris* in seed, post-treatment.

The PCR assay permits the sensitive detection of *X. campestris*, but does not offer a means to discriminate between the DNA from living and dead pathogens. Residual bacterial DNA present in seed following successful treatment for *X. campestris* could lead to a positive PCR result, even though there is no risk of disease. Therefore, we also employed a bioassay and selective plating to determine the effectiveness of each treatment regime, against control batches of untreated seed. Only NSCAA was employed in the selective plating experiments, as we considered this to be the most useful selective media for isolation of *X. campestris* from seed. The bioassay is a very useful gauge of the presence of *X. campestris* in seed, providing a true indicator of the persistence of the pathogen following treatment. However, this technique is extremely labour-intensive and time-consuming, and large quantities of seed need to be sown, particularly when confirming the successful treatment of seed carrying a low pre-treatment level of infection. These factors limited our ability to include low-level infection samples in the bioassays.

The highest infection rate samples (1/10) were assessed by all methods: PCR, selective plating and bioassay, and the germination rates of these samples were also determined. The results from the plating of seed washes onto selective media were consistent with all treatments leading to a reduction in the overall microbial load of a sample. *X. campestris* colonies were observed on NSCAA media from untreated seed washes and from seed treated with bleach and Incotec. However, the absence of any growth for seed washes from the copper-treated samples suggested that residual copper may be inhibiting microorganisms on the selective media. This notion was supported by the observation of a

blue tint in the washes of copper treated seed batches. The plating assay results indicated that the most effective treatment was copper>bleach>Incotec>untreated.

PCR analyses of seed wash extracts demonstrated that all of these samples contained *X. campestris* DNA. The bioassay confirmed the presence of black rot in seven of the eight samples; one of the copper-treated replicates was black rot-free in the bioassay. The DNA extract used in the PCR assay was derived from a sample of 8 000 seeds, whereas the bioassay included only 1 000 seeds. This discrepancy may reflect the amplification of small quantities of residual DNA from a successfully treated (black rot-free) seed batch, or alternatively, the treatment may have reduced the infection rate such that it was not detected in 1 000 seeds but was still present in a sample of 8 000 seeds. Although complicated by the inhibitory effect the treatments had on the amplification reaction, the results of the PCR assay suggest that none of the treatments eliminated the black rot infection.

Despite the prolonged use of copper-containing compounds to control the bacterium in *Brassica* field crops, copper resistance does not appear to have emerged in either Australian or foreign isolates of *X. campestris*, a finding consistent with a recent study of QLD field isolates (Martin *et al*., 2003). The maximum $CuSO_4$ concentration tolerated by the *X. campestris* strains surveyed, 0.2 mM, is considered to reflect the sensitivity of these isolates to copper. The thresholds reported for determination of isolates as resistant, intermediate or sensitive varies for different species and studies; *Pseudomonas syringae* isolates exhibiting a tolerance ≥0.32 mM $CuSO_4$ are deemed to be resistant by Scheck *et al*. (1996), whereas Tesoriero *et al*. (1997) classify *Pseudomonas syringae* pv. *tomato* isolates growing on <0.6 mM, 0.6-1.2 and >1.2 mM $CuSO_4$ to be sensitive, tolerant (intermediate) and resistant, respectively. Copper resistance, defined as tolerance ≥0.8 mM $CuSO_4$, has been occurring in *X. axonopodis* pv. *vesicatoria* (formerly *X. campestris* pv. *vesicatoria*) for at least 20 years (Gore and O'Garro, 1999). Resistance in both genera is plasmid-encoded, and horizontal exchange of resistance genes is believed to have occurred (Cooksey, 1990). Whilst it is fortunate that *X. campestris* field isolates are sensitive to copper, there is certainly the potential for resistance to develop, particularly via the acquisition of copper resistance plasmids. Vigilance is necessary to ensure the future efficacy of copper-based control measures against *X. campestris*.

CHAPTER 6

Technology transfer

INTRODUCTION

The project was publicised through articles in several industry journals: Good Fruit and Vegetables, Brassica IPM, VegieBites and Agriculture Today, resulting in new industry contacts and vital access to black rot-infected seed and leaf samples. We were also approached by several European seed companies interested in the detection assay, and a subsequent collaboration was formed with the French company Vilmurin, Clause and Cie (VCC), enabling us to exchange cultures and validate the method against foreign isolates of verified pathogenicity status, which are not available through the international culture collections.

The "PCR-based detection of *Xanthomonas campestris* pathovars" workshop was held 1st to 3rd December 2003 at Elizabeth Macarthur Agricultural Institute, to transfer the molecular testing technology to stakeholders. Participants were from state departments of agriculture (Victoria, Queensland, Western Australia and NSW) and from Enza Zaden (Australia) Pty Ltd. Participants in the largely practical workshop were given the opportunity to evaluate the new method in the laboratory, detecting *X. campestris* from Brassica seed, black rot lesions on infected seedlings, and bacterial colonies on agar plates. Even those with minimal previous experience in PCR techniques were rewarded with a successful result at the end of their laboratory work. Both the organisers and participants found the workshop an extremely worthwhile experience, enabling attendees to foster relationships with their interstate counterparts and take part in valuable scientific discussion. Evaluation of the intensive workshop indicated that it was a resounding success and all participants thought it highly likely that they would recommend the testing to clients and/or that their agency offer the test method themselves, under license.

The contacts developed as a result of the workshop were directly beneficial to the ongoing project; *X. campestris* isolates collected during a previous Queensland DPI project were made available to us, allowing the *hrpF* PCR assay to be validated against many more recent Australian field isolates than would have been possible otherwise, and representatives of Enza Zaden (Australia) Pty Ltd offered valuable technical advice regarding disinfection methodology.

WORKSHOP SCHEDULE

DAY 1

2.00 pm	Seminar	Welcome and Introduction to EMAI – Dr Paul Arthur, Institute Supervisor.
2.15 pm		Overview of project and assay design "VG01024 Quality assurance for improved management of black rot in brassicas" by Dr Deborah Hailstones, Molecular Biologist / Mr Len Tesoriero, Plant Protection Officer.
2.45 pm		PCR-based assay to detect pathovars of *Xanthomonas campestris* by Dr Tracey Berg, Project Officer.
3.30 pm		Discussion
4.00 pm	*Afternoon tea*	
4.30 pm	Tour of EMAI	Ms Nancy Kelly, Plant Health Diagnostic Service Co-ordinator.
6.00 pm	*Dinner in Camden*	

DAY 2

8.30 am	Laboratory	Set up seed washes
		Prepare samples from lesions and colonies
10.15 am	*Morning tea*	
10.45 am	Seminar	PCR protocol
12.00 pm	Laboratory	Begin extraction of DNA from seed washes (lyse cells over lunch)
12.45 pm	*Lunch*	
1.30 pm	Laboratory	Complete DNA extraction and template dilution
2.30 pm	Laboratory	Set up PCR reactions to cycle overnight
6.00 pm	*Dinner in Camden*	

DAY 3

8.30 am	Laboratory	Agarose gel electrophoresis (and demonstration of real time PCR)
10.15 am	*Morning tea*	
10.45 am	Laboratory	Stain gel
11.00 am	Laboratory	Capture gel image
11.15 am	Seminar	Interpretation of results, discussion
12.30 pm	*Lunch*	

WORKSHOP : EVALUATION BY ATTENDEES

Seminar sessions of Monday 1st December

The number of responses (out of 5) to each choice is given below, or in brackets following comments:

1. How would you rate Deb's overview of the project and the development of the molecular test?

Poor	fair	good	very good
0	0	1	4

2. Do you think this presentation was appropriate in terms of content, detail, assumed knowledge etc? Any other comments?
   - *Great and aimed at exactly the right level.* (1)
   - *Clear explanations of techniques and terms used. Perhaps more pathology content /the purpose or practical applications of the technique.* (1)
   - *Very appropriate – I haven't done this stuff for some time, but it was pitched at the right level for me.* (1)
   - *Excellent. Could do with an easier intro to PCR – animation?* (1)
   - *Yes, appropriate.* (1)
3. How would you rate Tracey's detailed presentation on the molecular method?

poor	fair	good	very good
0	0	0	5

4. Do you think this presentation was appropriate in terms of content, detail, assumed knowledge etc? Any other comments?
   - *Great and aimed at the right level.* (1)
   - *Yes.* (1)
   - *Great.* (1)
   - *Excellent. Could do with an easier intro to PCR – animation?* (1)
   - *The presentation was great but it was a lot of information to absorb in one hit.* (1)
5. Do you feel that these presentations gave you enough background to confidently undertake the laboratory sessions that followed?

   Yes. (3)

   Yes, with supervision in the lab (1)

   Yes, because anything that was left out was covered later. (1)

6. How would you rate Nancy's tour of EMAI, including the PC3 facility? *(originally intended to include glasshouses & PHDS; PHDS was covered the next day)*

irrelevant	good	useful	interesting
1		1	3

7. Was there any other area of EMAI that you would like to have seen in this tour?
   - *Glasshouses* (4) – this was omitted due to rain, then time constraints
   - *Other molecular labs on site* (1)

Laboratory sessions of Tuesday 2nd and Wednesday 3rd December

8. How would you rate the laboratory sessions, in general?

poor	fair	good	very good
0	0	0	5

9. Do you think the sessions were sufficiently well organised (eg support provided by the team, detail in the manual, provision of reagents and equipment etc)?

- *Yes/excellent* (5)

10. Was Tuesday's "tutorial" (the more detailed discussion of the practical procedures to be followed in the afternoon) helpful in preparing you for these activities?
    - *Yes/excellent* (5)
11. Any other comments?
    - *Great job, well done.* (1)
    - *Any hands-on with novices will be slow, may be able to start lab session (PCR setup) earlier.* (1)
    - *Workshop was excellent, learned a lot.* (1)

Your impressions

13. How would you rate the workshop, overall?

poor	fair	good	very good
0	0	0	5

14. Is your agency/company equipped to perform this testing procedure?

no	yes, but…	yes
1	1	3

    - *Yes, but… those with molecular capabilities in my agency are not into vegetables.*

15. Now that you have seen the details of this testing procedure, and assuming that your agency/company had the equipment and experience to do so, do you think that you would support its implementation by your agency/company?

no	unlikely	possibly	definitely	not sure
0	0	2	3	0

16. Would you recommend your company/agency ask another organisation (NSW Ag or someone else) to perform this testing on their behalf?

no	unlikely	possibly	definitely	not sure
0	2	2	1	0

17. How would you rate your own experience with molecular methods, prior to this workshop?

non-existent	poor	fair	strong	very strong
1	1	2	0	1

18. Any other comments?
    - *I liked the hands-on, even though it was very time consuming, it builds the individual's confidence in undertaking the test in their own lab.* (1)
    - *Well organised and extremely well run.* (1)
    - *Great idea to have this workshop – very good to try to extend this technique to other labs/companies where it may be used in a practical way – there should be more of this in molecular biology.* (1)
    - *Very enjoyable workshop – good to network with other similar agencies in other states.* (1)

Left to right: Len Tesoriero, Jane Ray, Tracey Berg, Deb Hailstones, Heidi Martin, Debbie Lydon, Dan Trimboli, Stacey Azzopardi, Stephen Doughty.

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Workshop co-ordinators

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Recommendations

BLACK ROT MANAGEMENT STRATEGIES

Seed detection and disinfection

Batches of seed should be tested for *X. campestris* using the PCR detection assay developed in this project, or at a minimum by a seedling bioassay. Growers should insist on pathogen-tested seed when considering their purchase.

Where seed is infected, seed companies have the option of using any of the disinfection methods assessed in this project. Although none of the seed disinfection methods completely eliminated all bacteria, it is known that potential spread and speed of disease development is related to the initial inoculum level (Kocks *et al.*, 1999). Of the treatments studied in this project, copper acetate at 40 ^{o}C appeared to be superior despite having the greatest negative effect on percentage germination. Further studies are required to optimise this treatment, such as regimes at higher temperatures or in combination with exposure to low dose ultraviolet light-C, which has been shown to induce resistance in cabbage to black rot (Brown *et al.*, 2001).

Seedling Production

Strategies to decrease the development and spread of black rot during seedling production are essential to avoid potential losses after transplantation. Following are best practice strategies to minimise the risk of black rot in seedling production:

- The use of pathogen-tested and treated seed (as discussed above) is a primary step to avoid disease incidence.
- Seedling trays should be effectively cleaned and sanitised prior to use, particularly where they are reused. High-pressure washing followed by chlorination (or use of chlorine dioxide), or autoclaving trays is recommended.
- Physical separation of different seed batches and cultivars will reduce the risk of bacterial dispersal. This can be important where seedlots of unknown black rot status are being grown at the same time as pathogen-tested material. An exclusion zone of >6metres has been shown to minimise the risk of pathogen dispersal from a point source of infection (Kocks *et al.*, 1999).
- As *X. campestris* is spread from guttation droplets on leaf margins, seedlings should not be watered when guttation droplets are apparent. This may mean delaying overhead irrigation until later in the day, which also permits leaves to dry. Such a strategy is most important if seedlings are being handled or moved. Avoid any unnecessary handling or mechanical contact that moves moisture films from plant to plant. During wet weather conditions, disease spread

is favoured and greater care is required when handling plants. Future studies could determine if there is any advantage in using low doses of disinfectants such as chlorine dioxide through overhead irrigation during these periods.

- Chemical controls are preventative and not curative. Copper sprays should protect leaves and reduce spread of the disease. The fact that all isolates tested in this study were sensitive to copper supports the expected efficacy of this treatment. Thorough coverage of plants is required for protective chemical sprays. A single application of phosphonic acid (also known as phosphorous acid) which is chemically related to the systemic fungicide, fosetyl-aluminium (fosetyl-Al), may also be useful to reduce spread of this disease. Mochizuki and Alvarez (1996) showed that a single prophylactic treatment (spray or drench @ 4.8g/L aqueous solution) of fosetyl-Al reduced the invasiveness of *X. campestris* on inoculated cabbage seedlings. The effect was almost immediate and persisted for 21 days. This effect was more pronounced at 20^{o}C and decreased as temperature increased. Phosphonic acid is commonly used for control of downy mildew on brassicas and a permit for this use against black rot should be sought if these effects can be validated.
- Control insect pests such as aphids, fungus gnats (sciarid flies) and shore flies, which can spread bacteria.

Field Production

Following are suggested strategies to minimise black rot incidence and spread in the field:

- Upon delivery examine seedlings carefully for symptoms of black rot (and other diseases). Note any V-shaped lesions extending in from leaf margins, particularly any blackening of veins associated with lesions. Suspect plants should be carefully removed, sealed in plastic bags and sent to a diagnostic laboratory for confirmation. Notify the seedling producer immediately of any disease concerns (they may request a sample for independent diagnostic testing). Remember seedling infection can also be symptomless, so make sure you have confidence in the production standards of your seedling producer and insist that the seed used has been tested for black rot.
- Avoid planting into ground where the disease has occurred within two years. This is because *X. campestris* can survive in plant residues for at least six months (Schaad & White, 1974) and in soil for up to fourteen weeks (Dane & Shaw, 1994). Furthermore, these bacteria can survive epiphytically for several weeks on non-hosts such as tomatoes and lettuce. Crucifer weeds may also harbour infective bacteria. Arias *et al.* (2000) demonstrated that high soil moisture levels reduced *X. campestris* survival and hastened breakdown of crop residues.

Further research could explore the potential benefits of adding composted organic matter or formulated cellulolytic bacteria that may facilitate this process.

- Use of resistant *Brassica* cultivars may be feasible in a field where disease has previously occurred, providing the race of *X. campestris* is known. This would require the isolation of bacteria from infected plant material of the previous crop. Bacteria can then be inoculated to a set of differential indicator plants to determine the race. The molecular assay described in this report can not distinguish races.
- Ensure machinery used for transplanting and cultivation has been cleaned and sanitised, particularly if the disease has been previously recorded on the farm in recent times.
- Avoid overhead watering where possible.
- Wait until plants are dry before working in crops.
- Control pests as described above for seedlings.
- Use chemicals as detailed above for seedlings.

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