Final Report

Blackleg survey – English and Welsh seed crops 2013-2015

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1. SUMMARY

Annual surveys conducted in England and Wales during the growing season identified blackleg in at least 23% of seed stocks entered for classification and established the cause in a large majority (86-94%) of affected seed stocks as *Pectobacterium atrosepticum* (*Pba*). Seed stocks with blackleg caused by *P. atrosepticum* were almost exclusively grown from seed of GB origin. *Pectobacterium carotovorum* subsp. *brasiliensis*, *Pectobacterium wasabiae* and a subgroup of *P. carotovorum* subsp. *carotovorum* were also confirmed for the first time in England and Wales as causes of blackleg amongst the remaining 6-14% of blackleg-affected seed stocks. *Dickeya solani* and *Dickeya dianthicola* were responsible for less than 2% of blackleg in these affected stocks. Although most cases caused by *Dickeya* species and *P. carotovorum* subsp. *brasiliensis* have been found in stocks grown from seed imported directly from the Netherlands, *Dickeya dianthicola* and *P. carotovorum* subsp. *brasilliensis* were found in stocks grown from seed of GB origin for the first time in 2015. *P. wasabiae* and the blackleg causing sub-group of *P. carotovorum* subsp. *carotovorum* appear to have been already distributed in seed produced in GB and elsewhere around Europe for many years.

A new variable number tandem repeat (VNTR) DNA typing scheme was developed, which distinguished at least 18 identifiable strains of *Pectobacterium atrosepticum* (*Pba*). This was used to further investigate sources and pathways of contamination during the first year field generation to PB-1 seed crops produced from the same mini-tuber stock at 3 locations in Scotland and England. Contamination of progeny tubers occurred during the first field generation from mini-tubers with *Pba* isolates that appeared to originate from the local environment. Sources of contamination most likely included blackleg plants from crops of lower grade growing in the vicinity. Planting mini-tubers in isolation from other seed and ware stocks at one location appeared to reduce the risk of contamination with *Pba* in each of the three years. Contamination levels on seed tuber stocks increased with subsequent field generations, with the sources of contamination being identified as carry-over latent infections on the planted seed stock and contaminated harvesting machinery, in addition to blackleg plants from neighbouring crops.

As a preliminary investigation into direct toxicity effects of different commercial haulm dessicants to *Pectobacterium atrosepticum*, a simple *in vitro* test showed that sulphuric acid and diquat were equally toxic, whereas carfentrazone was non-toxic, even when exposed to undiluted formulation.

2. INTRODUCTION

A survey of seed potato stocks in England and Wales has been conducted since 2010 with levy board support. Samples of infected plants from all stocks in which blackleg was observed by Plant Health Seed Inspectors (PHSI) during classification inspections are submitted to Fera for diagnosis. The results from previous surveys are summarised below:

Blackleg findings in seed potato stocks entered for classification in England and Wales (2010-2012).

	2010	**2011**	**2012**
% seed stocks with blackleg	32.1	21.5	33.8
% blackleg caused by *D. solani*	7.0	2.3	1.8
% blackleg caused by *D. dianthicola*	0.4	0.6	1.8
% blackleg caused by *P. atrosepticum*	75.2	74.4	84.1

To further establish the cause and extent of blackleg caused by soft rot bacteria other than *P. atrosepticum* and *Dickeya* spp., the same methodology was continued in 2013-2015 with the introduction of DNA barcoding methods (Parkinson *et al*., 2009) for accurate pathogen identification.

In an attempt to accurately identify sources and pathways of contamination of pre-basic seed crops with *Pba*, a new strain typing (VNTR) method was developed. This could then be used to analyse DNA of different *Pba* populations isolated from blackleg plants and progeny tubers in pre-basic seed stocks and compare them with isolates from the surrounding environment and on associated machinery. Investigations reported below included intensive monitoring of PB-1 crops grown from the same source of mini-tubers during the first field generations at different locations in England and Scotland. The same stocks were then followed through subsequent generations so the extent and pathways of contamination with *Pba* populations from different sources could be investigated.

3. MATERIALS AND METHODS

3.1. Blackleg surveys of England and Wales

Collection of plants with blackleg symptoms
Samples of blackleg plants were collected by APHA (Animal and Plant Health Agency) inspectors during routine seed certification inspections in 2013, 2014 and 2015 and mailed directly to Fera. At least one sample was provided for every seed stock entered for certification in England and Wales in which blackleg symptoms were observed during first or second field inspections. The samples were labelled in such a way that the grower or business was not identified, but further trace-back of the stock could be done for samples which tested positive but where the origin of the seed stock was not known.

Isolation and identification of pectolytic bacteria
Pectolytic bacteria were isolated from the leading edge of blackleg affected stems on double layer selective CVP-M medium. Pectolytic colonies were purified on PDA, NA and TSBA media and identified according to fatty acid profile, according to Stead (1992). Isolates identified as *Pectobacterium* or *Dickeya* spp. were further identified by *recA* sequence determination, as described by Parkinson *et al*. (2009) and by real-time PCR assays specific for *Pectobacterium atrosepticum*, *Dickeya solani* and *D. dianthicola* (Prichard *et al*., 2013).

3.2. Monitoring of high grade seed crops for *Pectobacterium atrosepticum*

A single mini-tuber clone was planted in each of 2013, 2014 and 2015, at up to 3 locations (Farm 1 in North Yorkshire and Farms 2 and 3 in Aberdeenshire), for commercial pre-basic seed production (classification to PB-1). Prior to planting in each year, soil samples were collected and pooled from 100 locations within the areas to be planted. Aliquots of serially-diluted suspensions of soil (10% wt/vol) were spread onto CVP-M medium before and after anaerobic incubation in equal volumes of double strength pectate enrichment medium at 21 °C for 48 hours. Pectolytic bacteria were purified after 24-48 hrs incubation at 28 °C on CVP-M.

Samples of 100 mini-tubers before planting and 100 of the PB-1 progeny tubers after harvest were collected at each location and tested by grinding heel-end cores and longitudinal strips of peel from the circumference of 5 subsamples (each of 20 tubers) in PB

buffer (pH 7.0) containing 0.1% sodium pyrophosphate as antioxidant. The resulting suspension was then spread on CVP-M medium for isolation of pectolytic bacteria. The tubers were also encouraged to rot by wrapping in moist paper towel in plastic bags at 21 °C after wounding lenticels with sterile toothpicks. Isolations on CVP-M were made from the margins of rots which developed after 48 hours.

The crops were visited regularly during the season and inspected together with adjacent potato crops growing in the same field for development of any blackleg symptoms. Any plants with blackleg symptoms were analysed for pectolytic bacteria as described above. In cases where no blackleg was observed, leaf samples from the top of the canopy and leaf debris from the soil surface were collected and analysed after washing in phosphate buffer and spreading aliquots of the washings onto CVP-M medium.

In each growing season, rain water was collected regularly in rain gauges positioned in the growing crops and tested for pectolytic bacteria by first concentrating by centrifugation at 10.000 g for 15 minutes and discarding the supernatant. The resulting concentrate was then spread onto CVP-M medium before and after incubation at 21 °C for 48 hours in pectate enrichment broth.

The surfaces of planters and harvesters were swabbed before and after contact with seed or progeny tubers. Swabs were incubated anaerobically in pectate enrichment medium at 21 °C for 48 hours. The medium was then spread onto CVP-M medium for isolation of pectolytic bacteria.

In 2014 and 2015, the following field generations from the original minituber-planted crops were also monitored in the same way. In addition, a number of high grade seed stocks of different varieties growing at the same locations were also monitored. Additional samples from high-grade commercial seed crops (grades PB 1-3) were also provided directly by pre-basic seed growers at 4 locations in Scotland (Farms 3-6). Samples of blackleg plants, harvested tubers and swabs from harvesters before and after lifting were tested as described above.

3.3. Development of variable number tandem repeat (VNTR) method for typing of *Pectobacterium atrosepticum* isolates.

The published genome sequence of *P. atrosepticum* SCRI 1043 was analysed for selection of tandemly repeated sequence motifs using the program mreps (Kolpakov *et al*., 2003). Repeat sequences which were less than five bases in length or were repeated less than five times or did not contain at least three different bases or which contained sequences of limited sequence diversity (e.g. single base repeats) were all excluded. Primers were designed to the sequence flanking 13 different tandemly repeated sequence motifs. These were fluorescently labelled and tested by PCR amplification from DNA extracted from 10 reference *Pba* isolates of diverse geographical origins and dates of isolation. Primers which consistently resulted in amplicons of discriminating sizes were selected for further analysis of *Pba* isolates collected during this project.

3.4. *In vitro* toxicity of desiccants

To investigate the toxicity of three different haulm desiccants to *P. atrosepticum* isolate SCRI 1043, an agar plate assay was developed. The products used were sulphuric acid (77%), diquat (Retro®) and carfentrazone (Spotlight®). Filter paper disks were soaked in decimal aqueous dilutions of each product and placed onto nutrient agar which had been

seeded with a high concentration of the bacteria before pouring into Petri plates. Inhibition of growth of the bacterium was determined according to zones of inhibition of growth of the bacteria around each treated disc after incubation of the agar plates at 28 °C for 48 hours.

4. RESULTS

4.1. Blackleg survey of England and Wales

The blackleg survey of seed stocks during field inspections in England and Wales was completed between June and September each year. Symptomatic plants were collected by APHA inspectors from around 23% of all of the seed stocks entered for classification in England and Wales in each of the three years. The results of laboratory testing at Fera (Table 1) confirmed that the majority of the blackleg (86-94%) over the three years was caused by *Pectobacterium atrosepticum* (*Pba*). With the exceptions in 2013 of 4 stocks originating from the Netherlands and 1 stock each from Germany and Denmark, and in 2014 of 3 stocks originating from the Netherlands, 2 from Germany and one from France, most stocks with blackleg caused by *Pba* were of GB origin. In 2015, all blackleg caused by *Pba* was found in stocks of GB origin.

Table 1: Summary of blackleg findings in seed potato stocks entered for classification in England and Wales 2013-2015.

	2013	**2014**	**2015**
% seed stocks with blackleg	23.4	23.4	23.7
% blackleg caused by *P. atrosepticum*	86.5	93.6	89.4
% blackleg caused by *P. carotovorum* subsp. *brasiliensis*	5.5	3.4	0.8
% blackleg caused by *P. wasabiae*	3.8	1.8	7.4
% blackleg caused by *P. carotovorum* subsp. *carotovorum*	2.5	0.4	1.6
% blackleg caused by *D. solani*	1.7	0.4	0.4
% blackleg caused by *D. dianthicola*	0	0.4	0.4

Other causes of blackleg were *Dickeya solani* (in less than 2% of the total number of stocks with blackleg in each year) and *Dickeya dianthicola* (in less than 1% of the total number of stocks with blackleg in 2014 and 2015 only). All of the seed stocks infected with *Dickeya* spp. originated in the Netherlands, with the exceptions in 2015 of one finding of *D. solani* in a stock of French origin and one of *D. dianthicola* in a stock of GB origin.

In each year there were also a number of cases where *Pectobacterium* species other than *atrosepticum* were isolated from the blackleg plants. Further identification of these bacteria using *recA* gene barcode sequencing indicated that these isolates clustered into 3 clades (Figure 1). One clade, comprising isolates with identical recA sequence, was most closely related to the type strain of *P. carotovorum* subsp. *brasiliensis* (LMG 21371). Most of these isolates came from crops grown directly from seed of Netherlands origin, where this pathogen has been recently reported (Nunes Leite *et al*., 2014). However, some of these isolates were also obtained from stocks of GB and German origins. Another clade, comprising isolates from seed stocks of GB, Danish, German and Netherlands origins, was most closely related to *Pectobacterium wasabiae*, which has also recently been reported to cause blackleg disease and is thought to have been present in European potato for many years (Pasanen *et al*., 2013). The third clade, comprising isolates from seed stocks of GB and Netherlands origin, was recognised as a sub-group of *P. carotovorum* subsp. *carotovorum* (*Pcc*), which has also been recently associated with blackleg symptoms in Europe. It is probably also widespread in European potato and differs from another sub-group of *Pcc* which contains the type strain (NCPPB 312) and causes rotting of potato tubers but not blackleg symptoms in stems (Pasanen *et al*., 2013).

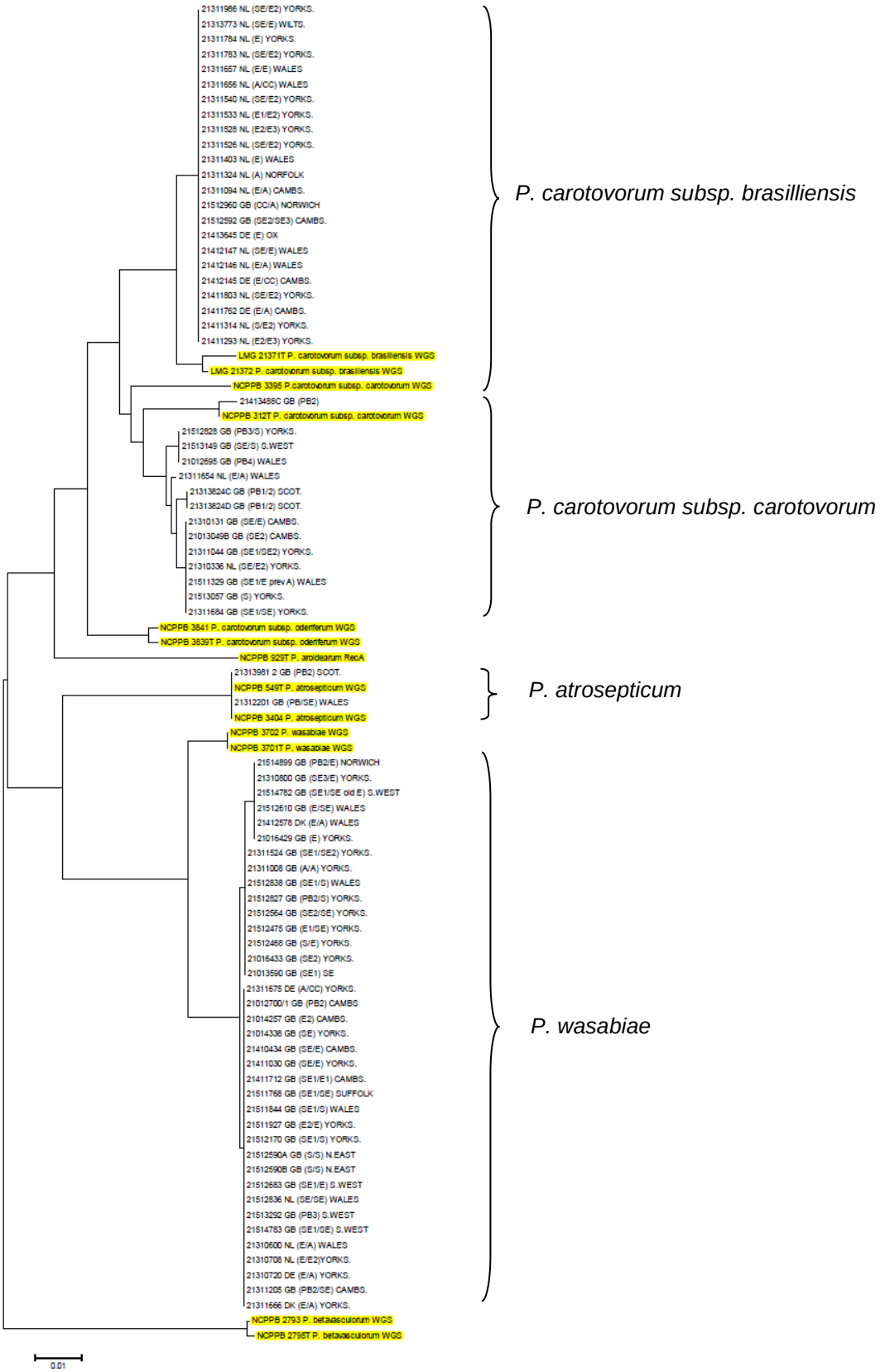

Fig. 1: Bootstrap consensus tree of forward and reverse recA sequences of strains belonging to *Pectobacterium* sp. isolated from blackleg symptoms collected during seed inspections in 2010, 2013, 2014 and 2015 but not originally identified as *P. atrosepticum*. Reference and type strains of *Pectobacterium* species and subspecies are highlighted in yellow (WGS = whole genome sequence available). Country of origin of the seed planted, grade of seed/expected grade of harvested crop and area where the seed crop was grown is also shown.

4.2. Development of variable number tandem repeat (VNTR) method for typing of *Pectobacterium atrosepticum* isolates.

Five primer sets were eventually selected from an initial set of 13 identified that amplified tandemly repeated sequences in the genome of *Pectobacterium atrosepticum* (SCRI 1043). Four VNTR profiles were initially identified amongst a panel of only 10 *Pba* reference potato isolates collected over a 60 year period (1939-99) from worldwide origins (Table 4).

The selected primers were then used to identify a further 14 VNTR profiles amongst the isolates collected from PB potato crops and environmental sources. Profile 1 was the most frequently found, occurring in all locations studied. This profile was also shared with other *Pba* reference strains isolated from potato in GB, including NCPPB 138 which was isolated as early as 1939. Profile 2 is also known to be present in GB since at least 1962 (NCPPB 1277) and was also found across all locations. Although most VNTR types were found at multiple locations, others (profiles 7, 13 and 16) were only found on individual farms.

Table 2: VNTR profiles amongst 18 reference isolates of *P. atrosepticum*

Profile	Isolate	Source	Year	Amplicon length (bp)				
				TR2	TR4	TR8	TR10	TR12
1	NCPPB 138 NCPPB 309 NCPPB 3390 NCPPB 4056	UK UK USA UK	1939 1951 1985 1999	298	275	264	278	242
2	NCPPB 432 NCPPB 435 NCPPB 1277	Israel Zimbabwe UK	1957 1957 1962	307	275	264	278	242
3	SCRI 1043	UK	1985	307	275	291	319	249
4	NCPPB 1743 NCPPB 3406	Brazil Canada	1965 1985	307	313	296	335	228
5	21318081 1.2	UK	2013	298	265	264	278	242
6	SHA2 2	UK	2013	298	265	296	310	242
7	1022/1/3	UK	2013	298	285	264	278	242
8	SHB4 2	UK	2013	307	255	264	278	242
9	J19/Jelly 17b	UK	2013	307	265	296/300	310	249
10	21320327/2/1	UK	2013	307	265	302	327	228
11	21313823-c	UK	2013	307	275	264	319	242
12	21321575/2/2	UK	2013			282	278	
13	21313981 Cab4	UK	2013	307	319	300	327	
14	21313981 Orc4	UK	2013	298	275	291	278	242
15	21413337	UK	2014	307	301	296	327	
16	21418146_2C	UK	2014	307	265	302	310	249
17	21418724_5C	UK	2014	298	275	291	319	242
18	21524914_C4	UK	2015			267	278	

4.3. Monitoring of high grade seed crops planted from the same clone of mini-tubers for PB-1 certification in England and Scotland.

Identification of *Pectobacterium* species isolated from samples taken during multiplication of seed stocks from mini-tubers indicated that the only detectable sources of *Pba* were blackleg plants which developed in stocks of lower grades growing in the same field as the crops being monitored (Table 3). These were more intensively monitored in 2014 and 2015 than in 2013, when only one blackleg plant was sampled in the Scottish location. *Pba* was not detected in soil before planting at either location in any year, nor was it ever detected in rainwater sampled immediately after rainfall, in leaf debris collected from the soil surface within the target crops or on planting or harvesting machinery coming into contact with the target crops.

Table 3: Monitoring of high grade seed crops planted from the same clone of mini-tubers for PB-1 certification in England and Scotland.

Location of crop	**Samples tested**	**Number of samples from which *Pba* was isolated**					
		2013		2014		2015	
		Before enrichment	After enrichment	Before enrichment	After enrichment	Before enrichment	After enrichment
England	Soil before planting	0/4	0/4	0/4	0/4	0/4	0/4
	Planter after planting	0/5	0/5	0/5	0/5	0/5	0/5
	Rain water	0	0	0/6	0/6	0/6	0/6
	Leaf debris	0/4	-	0/4	0/4	0/4	0/4
	Blackleg plants from neighbouring seed stocks	0	-	**25/25**	-	**15/15**	-
	Harvester before lifting	0/5	0/5	0/5	0/5	0/5	0/5
	Harvester after lifting	0/5	0/5	0/5	0/5	0/5	0/5
Scotland	Soil before planting	0/4	0/4	0/4	0/4	0/4	0/4
	Rain water	0	0	0/3	0/3	0/3	0/3
	Leaf debris	0/3	-	0/4	0/4	0/4	0/4
	Blackleg plants from neighbouring seed stocks	**1/1**	-	**1/2**	-	**13/14**	-
	Harvester before lifting	-	-	0/5	0/5	0/5	0/5
	Harvester after lifting	-	-	0/5	0/5	0/5	0/5
Total no. *Pectobacterium* isolates tested:		18	47	26	110	28	90

Pba was not detected in any of the samples of mini-tubers taken before planting in each season and no blackleg symptoms developed in any of the crops grown from mini-tuber stocks in any of the 3 years. However, *Pba* was detected on harvested (PB-1 certified) progeny tubers of some of these crops at 2 of the 3 locations (Table 4). In 2013 and 2014, *Pba* contamination of PB-1 certified progeny tubers was detected at both Farm 1 in England and Farm 2 in Scotland. In 2015, *Pba* was not detected at Farm 1 on the PB-1 certified progeny. The mini-tuber stock was not planted at Farm 2 in 2015. At Farm 3 in Scotland, no contamination of the PB-1 certified progeny tubers by *Pectobacterium* species was detected in any of the three years. At this location, stocks grown from mini-tubers were planted in isolation from the other grades.

Table 4: Contamination of high grade seed potatoes, grown from the same mini-tuber stocks in 3 locations, with *Pectobacterium atrosepticum* varying in VNTR profiles.

Farm location	Year mini-tubers planted	2013 Blackleg plants	2013 Harvested tubers	2014 Blackleg plants	2014 Harvested tubers	2015 Blackleg plants	2015 Harvested tubers
Farm 1: England	2013	0	12/33	2/2	11/11	7/7	7/7
	2014			0	1/20	2/2	0
	2015					0	0/5
Farm 2: Scotland	2013	0	13/15	0	15/20	NP	NP
	2014			0	3/23	NP	NP
	2015					NP	NP
Farm 3: Scotland	2013	0	0	0	8/17	4/4	7/7
	2014			0	0	0	0
	2015					0	0

VNTR profiles: 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18

NP = not planted

Table 4 also shows findings of *Pba* in subsequent field generations when the same stocks were multiplied in 2014 or 2015. Second field generation stocks certified at PB-2 in 2014 were found to be contaminated with *Pba* at all three locations. The third field generation stocks certified at PB-3 in 2015 at Farms 1 and 3 were also contaminated with *Pba*. Second field generation stocks certified at PB-2 in 2015 at Farms 1 and 3 remained free from detectable *Pba* contamination.

VNTR profiles of the *Pba* isolated from the harvested tubers varied with the location at which the mini-tubers were grown (Table 4), indicating localised sources and pathways of *Pba* infection. In second and third field generations, VNTR types amongst *Pba* isolates from harvested progeny tubers were sometimes but not always common to those observed on the planted seed.

Comparison of the VNTR profiles observed amongst *Pba* isolates from blackleg plants and progeny tubers of other seed stocks and grades growing in the same fields as those studied above is shown in Table 5. VNTR profiles amongst isolates from progeny tubers did not necessarily correspond with those amongst isolates from blackleg plants within the same stock. In 2014 and 2015, when more neighbouring stocks were monitored, there was some evidence at Farm 1 in England that the same few *Pba* VNTR types were causing blackleg regardless of the seed stocks (PB-1 and PB-2) studied, and that the same types were found in abundance on stocks which were downgraded due to high blackleg incidence (Variety 10 SE-2 in 2014 and Variety 7 PB-2 in 2015). The same VNTR types also appeared on harvested tubers together with some additional types for which a possible source was not determined. In contrast, however, VNTR types causing blackleg at Farm 3 in Scotland seemed to vary more between the PB2 and PB3 stocks studied, especially in 2015.

Table 6 shows data from the testing of additional samples from commercial crops supplied by pre-basic seed growers on 4 different farms in Scotland. Again, the VNTR profiles of *Pba* isolates from progeny tubers after harvest did not always correspond with those of isolates from blackleg plants within the same stocks. *Pba* was recovered from harvesting machinery in only one of the three years (2013), with *Pectobacterium carotovorum* subsp. *carotovorum* (*Pcc*) being predominant in the other years. Where Pba was recovered from the harvesters after lifting, the VNTR profiles did tend to match those of isolates from the blackleg plants within the same stocks.

In general, the combination of VNTR profiles identified amongst *Pba* isolates was unique to each location. Of the 18 recognised VNTR profiles, some (e.g. profiles 1, 2 and 5) were commonly found in most locations, whereas others (e.g. profiles 7, 13 and 16) were confined to only one location. Three profiles (4, 11 and 15) were found only in reference isolates from the Fera NCPPB culture collection and were not found amongst isolates from samples collected during this project.

Table 5: Proportion of *Pectobacterium* species identified as *P. atrosepticum* and their associated VNTR profiles amongst isolates from blackleg plants and harvested tubers of high grade seed stocks originating from the same mini-tuber stocks or from neighbouring seed stocks grown at the same locations.

Year	Location	Seed stock and grade obtained after harvest.	Blackleg plants		Harvested tubers	
			Proportion of Pba	VNTR profiles	*Proportion of Pba*	VNTR profiles
2013	England Farm 1	Variety 1 PB-1	0		12/33	
		Variety 2 PB-1	0			
	Scotland Farm 2	Variety 1 PB-1	0		13/15	
		Variety 3 PB-2	1/1		3/5	
	Scotland Farm 3	Variety 1 PB-1	0		0	
		Variety 4 PB-2	5/5		11/11	
		Variety 5 PB-2	5/5		12/12	
		Variety 6 PB-3	5/5		13/13	
2014	England Farm 1	Variety 1 PB-1	0/0		1/20	
		Variety 7 PB-1	0/0		7/16	
		Variety 8 PB-1	0/0		4/6	
		Variety 1 PB-2	2/2		11/11	
		Variety 8 PB-2	0/0		3/6	
		Variety 2 PB-2	3/3		NT	
		Variety 9 PB-2	1/1		10/15	
		Variety 10 SE-1	2/2		NT	
		Variety 10 SE-2*	17/17		12/12	
	Scotland Farm 2	Variety 1 PB-1	0/0		3/23	
		Variety 1 PB-2	0/0		15/20	

Table 5 (cont.):

Year	Location	Seed stock	Blackleg plants		Harvested tubers	
			Proportion of Pba	VNTR profiles	*Proportion of Pba*	VNTR profiles
2014	Scotland Farm 3	Variety 1 PB-1	0/0		0/0	
		Variety 1 PB-2	0/0		8/17	
		Variety 4 PB-2	5/5		21/22	
		Variety 11 PB-2	1/1		17/17	
2015	England Farm 1	Variety 1 PB-1	0		0/5	
		Variety 1 PB-2	2/2		0	
		Variety 1 PB-3	7/7		7/7	
		Variety 12 PB1	0		2/9	
		Variety 12 PB2	0		0/4	
		Variety 7 PB2*	5/5		4/4	
		Variety 2 PB2	1/1		1/2	
	Scotland Farm 3	Variety 1 PB-1	0		0	
		Variety 1 PB-2	0		0	
		Variety 1 PB-3	4/4		7/7	
		Variety 13 PB-2	1/1		3/3	
		Variety 14 PB-2	1/1		6/6	
		Variety 12 PB-3	2/2			
		Variety 15 PB-2	0/3		11/12	
		Variety 2 PB-2	1/1			
		Variety 16 PB-1	1/1		4/9	
		Variety 17 PB-2	1/1		11/11	

*Downgraded due to excess blackleg during inspection

VNTR profiles: 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18

Table 6: Proportion of *Pectobacterium* species identified as *P. atrosepticum* and their associated VNTR profiles amongst isolates from blackleg plants, harvesting equipment and harvested tubers of high grade seed stocks grown at different locations.

Year	Location	Seed stock	Blackleg plants		Harvester before lifting		Harvester after lifting		Harvested tubers	
			Proportion of *Pba*	VNTR profiles	Proportion of *Pba*	VNTR profiles	Proportion of *Pba*	VNTR profiles	Proportion of *Pba*	VNTR profiles
2013	Scotland Farm 3	Variety 4 PB-2	5/5						5/5	
		Variety 5 PB-2	5/5						5/5	
		Variety 6 PB-3	5/5		5/5		5/5		5/5	
	Scotland Farm 4	Variety 18 PB-2	5/5		2/8		4/12		6/17	
	Scotland Farm 5	Variety 19 PB-2	12/12		3/13		9/13		9/21	
	Scotland Farm 6	Variety 20 PB-2	3/5		0/4		0/4		7/25	
2014	Scotland Farm 3	Variety 4 PB-2	5/5		0/4		0/4		21/22	
		Variety 11 PB-2	1/1						17/17	
	Scotland Farm 4	Variety 2 PB-2	5/5		0/12		0/13		12/16	
	Scotland Farm 5	Variety 21 PB-2	0/5		0/7		0/14		0/10	
	Scotland Farm 6	Variety 17 PB-2*	5/5						14/14	
2015	Scotland Farm 3	Variety 13 PB-2	1/1		0/3		0/9		3/3	
		Variety 14 PB-2	1/1						6/6	
		Variety 12 PB-3	2/2							
		Variety 15 PB-2	0/3						11/12	
		Variety 2 PB-2	1/1							
		Variety 16 PB-1	1/1						4/9	
		Variety 17 PB-2	1/1						11/11	

Table 6 (cont.)

	Scotland Farm 4	Variety 22 PB-2	5/5		0/9		0/9		5/6	
		Variety 23 PB-2	1/1						5/5	
		Variety 24 PB-2	1/1						1/1	
	Scotland Farm 5	Variety 21 PB-2	3/3		0/5		0/5		1/7	
		Variety 19 PB-2	1/1						2/6	
		Variety 25 PB-2	1/1						0/5	
	Scotland Farm 6	Variety 15 PB-3	5/5		0/6		0/5		6/7	

*Downgraded due to excess blackleg during inspection

VNTR profiles: 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18

4.4. Contamination of progeny tubers of high grade seed stocks

The populations of *Pectobacterium* spp. detected on progeny tubers after harvest of the various pre-basic seed stocks in each season is shown in Tables 7 and 8 and summarised in Fig. 2. No *Pectobacterium* was detected on mini-tubers of the intensively monitored variety 1 before planting in any of the 3 seasons. However, contamination of the progeny tubers was observed following the first field generation at two of the three locations (Farms 1 and 2) where the mini-tubers were planted in 2013 and 2014, whilst they remained free from contamination at the third location (Farm 3) in each of the three years. Only one instance was recorded (at Farm 3 in 2014) where progeny tuber contamination was not detected following a second field generation. *Pectobacterium* populations detected on progeny tubers tended to increase with subsequent field generations, although the mean populations detected usually remained at low levels (10^1-10^3 cfu per g peel), widely accepted to represent a low risk of disease. Only one crop of PB-3 at Farm 1 exceeded a contamination level of 10^4 cfu per g peel.

Table 8 shows the variation of progeny tuber contamination levels across varieties within locations and seasons. Whilst contamination of many pre-basic stocks remained in the low disease risk category (less than 10^3 cfu per g peel) after the first field generation, some had reached the medium disease risk category (10^4-10^6 cfu per g peel) after only 2 field generations. Fig. 2 shows a significant increase in the overall mean contamination levels after the second field generation in 2013 and 2014 but not in 2015. Comparison of test results based on analysis of stolon-end vascular tissue cores or strips of peel taken longitudinally around the tuber circumference showed high levels of correlation between the two methods (Fig. 3). Although analysis of peel tended to give a slightly higher

contamination level, this did not significantly affect the disease risk category. Moreover, negative or very low findings of *Pectobacterium* in particular tuber stocks were usually reflected by both methods (Tables 7 and 8).

Table 7: *Pectobacterium* populations detected on pre-basic seed (Variety 1) grown from the same mini-tuber clones at 3 locations in England and Scotland.

Seed stock tested		Farm 1: England		Farm 2: Scotland		Farm 3: Scotland	
		Vascular cores	Peel	Vascular cores	Peel	Vascular cores	Peel
2013	Mini-tubers before planting	0	0	0	0	0	0
	PB1 harvested	$2.0x10^{0}$	$3.0x10^{1}$	$7.8x10^{2}$	$2.2x10^{1}$	0	0
2014	Mini-tubers before planting	0	0	0	0	0	0
	PB1 harvested	$2.8x10^{3}$	$5.4x10^{2}$	$1.0x10^{3}$	$1.2x10^{3}$	0	0
	PB2 harvested	$8.0x10^{0}$	$3.2x10^{3}$	$2.6x10^{3}$	$1.8x10^{3}$	$1.2x10^{2}$	$6.0x10^{1}$
2015	Mini-tubers before planting	0	0	0	0	0	0
	PB1 harvested	$3.3x10^{3}$	$1.0x10^{3}$	NP	NP	0	0
	PB2 harvested	$7.5x10^{3}$	$1.7x10^{3}$	NP	NP	0	0
	PB3 harvested	$1.0x10^{4}$	$2.0x10^{3}$	NP	NP	$7.5x10^{3}$	$1.5x10^{2}$

Table 8: *Pectobacterium* populations detected on other commercial pre-basic seed stocks at 6 locations in England and Scotland.

Location	Year	Stock	Vascular cores	Peel
Farm 1: England	2013	Variety 1 PB-1	NT	$1.0x10^2$
		Variety 2 PB-1	NT	$3.2x10^2$
	2014	Variety 1 PB-1	$2.8x10^3$	$5.4x10^2$
		Variety 12 PB-1	0	0
		Variety 7 PB-1	$1.3x10^3$	$5.0x10^3$
		Variety 8 PB-1	$7.0x10^1$	0
		Variety 26 PB-1	0	0
		Variety 8 PB-2	$2.2x10^3$	$2.9x10^1$
		Variety 1 PB-2	$8.0x10^0$	$3.2x10^3$
		Variety 9 PB-2	$2.0x10^3$	$4.6x10^1$
		Variety 10 SE-2	0	$2.6x10^1$
	2015	Variety 1 PB-1	$1.0x10^3$	$3.3x10^3$
		Variety 12 PB1	$1.1x10^3$	$2.0x10^3$
		Variety 1 PB-2	$1.7x10^3$	$7.5x10^3$
		Variety 12 PB2	$8.7x10^2$	$6.7x10^3$
		Variety 7 PB2	$1.5x10^3$	$1.0x10^4$
		Variety 2 PB2	$1.2x10^3$	$4.0x10^3$
		Variety 1 PB-3	$2.0x10^3$	$1.0x10^4$
Farm 3: Scotland	2013	Variety 1 PB-1	NT	0
		Variety 4 PB-2	NT	$7.2x10^3$
		Variety 5 PB-2	NT	$1.5x10^6$
		Variety 6 PB-3	NT	$1.0x10^4$
	2014	Variety 1 PB-2	$1.2x10^2$	$6.0x10^1$
		Variety 4 PB-2	$5.0x10^5$	$8.0x10^6$
		Variety 11 PB-2	$2.0x10^3$	$3.3x10^3$
	2015	Variety 1 PB-1	0	0
		Variety 16 PB-1	$3.2x10^3$	$9.0x10^3$
		Variety 1 PB-2	0	0
		Variety 13 PB-2	$2.8x10^3$	$1.1x10^4$
		Variety 14 PB-2	$2.6x10^3$	$1.1x10^4$
		Variety 15 PB-2	$2.8x10^3$	$1.1x10^4$
		Variety 17 PB-2	$2.2x10^3$	$1.1x10^4$
		Variety 1 PB-3	$1.5x10^2$	$7.5x10^3$
Farm 4: Scotland	2013	Variety 18 PB-2	NT	$3.9x10^4$
	2014	Variety 2 PB-2	$6.6x10^3$	$6.4x10^2$
	2015	Variety 22 PB-2	$2.9x10^2$	$1.7x10^2$
		Variety 23 PB-2	$5.0x10^1$	$4.2x10^3$
		Variety 24 PB-2	0	0
Farm 5: Scotland	2013	Variety 19 PB-2	NT	$1.9x10^6$
	2014	Variety 21 PB-2	$3.4x10^2$	$1.7x10^3$
	2015	Variety 21 PB-2	$1.1x10^4$	$3.7x10^2$
		Variety 19 PB-2	$2.4x10^3$	$8.1x10^1$
		Variety 25 PB-2	$2.7x10^4$	$2.0x10^4$
Farm 6: Scotland	2013	Variety 20 PB-2	NT	$2.4x10^6$
	2015	Variety 15 PB-3	$5.0x10^3$	$1.8x10^4$

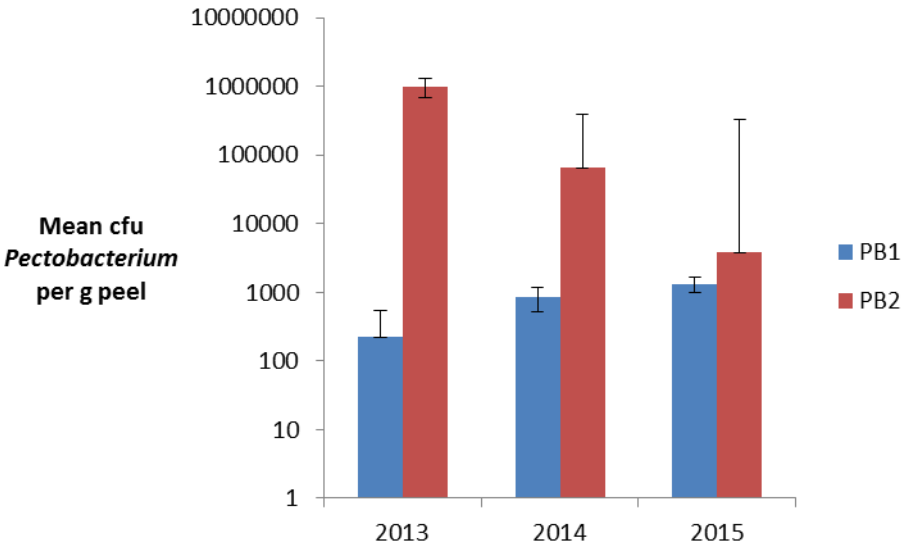

Fig. 2: Mean *Pectobacterium* contamination levels (cfu per g peel) on harvested progeny tubers of pre-basic seed determined after one (PB-1) or two (PB-2) field generations.

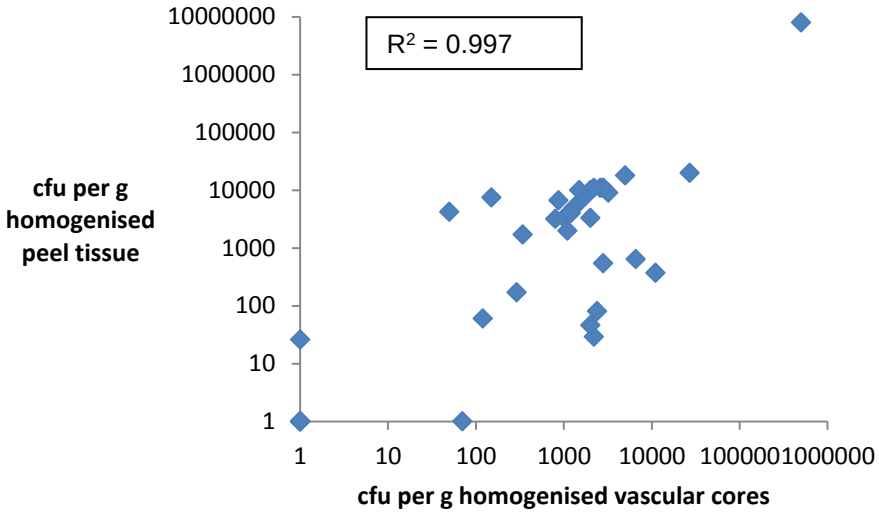

Fig. 3: Comparison of results from analysis of homogenised peel and stolon-end vascular core tissue, showing high correlation between *Pectobacterium* contamination levels determined by each method.

4.5. *In vitro* toxicity of desiccants

During *in vitro* agar plate tests, inhibition of the growth of *P. atrosepticum* (SCRI 1043) was observed by sulphuric acid (77%) and diquat (Retro®) when exposed either as undiluted product or diluted to 10% in water. Partial inhibition was observed when the products were diluted to 1% and no inhibition was observed at 0.1% or 0.01% or with water controls. Carfentrazone (Spotlight®) was not inhibitory at any concentration, even when exposed to the undiluted product.

Table 9: Effect of haulm dessicants on growth of *P. atrosepticum* (SCRI 1043) on nutrient agar at 28 °C.

Desiccant	100%	10%	1%	0.1%	0.01%	0
Sulphuric acid (77%)	-	-	(+)	+	+	+
Diquat (Retro®; Syngenta Ltd.)	-	-	(+)	+	+	+
Carfentrazone (Spotlight®; Belchim Crop Protection NV/SA)	+	+	+	+	+	+

\+ Growth of *Pba*,
(+) Trace growth of *Pba*,
\- No growth of *Pba*

5. DISCUSSION

The results of this and the associated project 114R475 have clearly confirmed that the majority of blackleg in seed stocks of GB origin is caused by *Pectobacterium atrosepticum* (*Pba*). In England and Wales, *Pba* accounted for 86-94% of blackleg found amongst all stocks entered for classification in the years 2013-2015. In addition, newly available DNA barcoding methods have allowed identification of other *Pectobacterium* species causing blackleg symptoms amongst the other 6-14% of cases of the disease investigated. These include 2 subspecies of *Pectobacterium carotovorum*; a subgroup of *P. carotovorum* subsp. *carotovorum* and a strain of *P. carotovorum* subsp. *brasiliensis*. Both have been recently shown to be causing blackleg in European potato (Pasanen *et al*., 2013; Nunes Leite *et al*., 2014) and the latter has mainly been isolated in 2013 from crops grown directly from seed imported from the Netherlands. A third species (*Pectobacterium wasabiae*) was also found to be causing blackleg in crops grown from seed of Danish, German and Netherlands as well as GB origins. This represents the first report of these pathogens causing blackleg in England and Wales. The recent widespread findings of both the subgroup of *P. carotovorum* subsp. *carotovorum* and *P. wasabiae* suggest that both pathogens have been circulating in European potato for many years. The strain of *P. carotovorum* subsp. *brasiliensis* has been recognised more recently and may not yet be as widely distributed as *P. atrosepticum* and the two subspecies of *P. carotovorum*.

As in previous years, the principal source of *Dickeya solani* and *D. dianthicola* was infected seed imported directly from the Netherlands. The low incidence of *Dickeya spp.* observed amongst seed stocks (less than 2% of blackleg affected stocks) again indicated that this pathogen is not a major cause of blackleg amongst seed multiplication in England and Wales. *Dickeya dianthicola* was, however, detected on a seed stock of GB origin in 2015, indicating the potential for its spread from stocks of non-GB origin for the first time during the current studies.

Intensive monitoring of a stock (Variety 1) of highest grade (certified to PB-1) seed potatoes grown in 3 locations in England and Scotland confirmed that contamination can occur within the first field generation. Initial contamination was seen to occur at low level and low frequency, sometimes only being detected after sample enrichment and not consistently detected across all sub-samples. It was also possible to find contamination in tuber samples from some first field generation stocks but not in those from neighbouring stocks of the same grade grown in the same environment. In these cases, it is possible that low and dispersed populations at or below the detection threshold were missed by the sampling and testing methods used. Higher contamination levels detected in subsequent field generations were more uniformly dispersed, resulting in more consistent results across samples and sub-samples.

Increased contamination in subsequent field generations may result from multiplication of any contaminating systemic bacterial populations in the stock, exposure to additional inoculum in the field following re-planting and/or transmission between infected and healthy tubers during handling. The only consistently detected sources of *Pba* inoculum in the field environment were plants with blackleg, most frequently occurring in stocks of lower grade, growing in the vicinity of the first generation stocks. Despite intensive sampling of soil before planting and rain water and leaf litter collected during the season, no *Pba* was recovered from any of these samples, even after enrichment to boost growth of any low populations.

The newly developed VNTR typing procedure has indicated the presence of multiple VNTR profiles within *Pba*, allowing comparison of isolates from different sources during monitoring of first generation contamination. Results show that crops grown from the same mini-tuber stock (Variety 1), which had tested negative for *Pectobacterium* before planting at different locations, produced tubers which were contaminated by *Pba* populations with different VNTR profiles in each location. This not only rules out the mini-tubers as the source of contamination in these cases, but supports the theory that first generation contamination originates from the local environment where the mini-tubers are planted. Furthermore, *Pba* isolates obtained from harvested tubers of subsequent field generations of the same Variety 1 stocks did not necessarily maintain the same VNTR profiles as those originally found in the first generation crop. This again indicates the importance of additional *Pba* contamination entering from the local environment and not necessarily systemically transmitted from generation to generation via the seed.

Other results have shown the occurrence of isolates from first field generation progeny tubers with the same VNTR profiles as isolates from blackleg plants sampled in the same fields from lower grade stocks with high disease incidence. The hypothesis that this contamination originated from nearby lower-grade stocks containing blackleg plants harbouring *Pba* with matching VNTR profiles is strongly supported by independent results from project 114R475 in which marked *Pba* strains have been shown to spread from infected bait plants to contaminate adjacent plots of first generation seed grown from healthy mini-tubers. It may also be relevant that in one location (Farm 3), where mini-tubers were reportedly planted in isolation from other seed stocks, no *Pba* was detected on harvested tubers of the first field generation (Variety 1 classified PB-1) stocks in any of the three years of this study.

The finding of *Pba* on swabs from harvesting machines on three Scottish farms in 2013 also supports the theory that healthy stocks can be contaminated by contact during machine handling. It is relevant that VNTR profiles of isolates from the harvesters matched those of blackleg plants previously collected from the harvested crops, indicating that the same bacteria were being spread from the crop via the machinery. However, it was also evident that VNTR types found on the harvested progeny tubers (in these cases PB-2 or PB-3 grade) did not all match those found on the harvesters, suggesting that other sources of contamination were also involved. For example, there were also VNTR matched profiles amongst *Pba* isolates collected from blackleg plants sampled from neighbouring seed stocks. *Pba* was not detected in any of the three years on harvesting machines following lifting of the intensively monitored crops of Variety 1 grown from mini-tubers. Since tuber contamination levels in these stocks were low and no blackleg plants were detected in the crops, it is possible that a lack of any rotting haulm or tuber material in the lifted crop was the reason for no detectable contamination of the machinery. This may not have been the case for the lower grade stocks where harvester contamination was observed. The reason why *Pba* contamination was detected on harvesting machines in only one of three years is not yet understood. In 2014 and 2015, contamination was predominantly by *Pectobacterium carotovorum*, which may have masked the presence of

any lower populations of *Pba*. The change in predominance of *Pectobacterium* species may simply be a reflection of the prevailing conditions during the harvesting period.

A simple *in vitro* study demonstrated that the haulm dessicant diquat was essentially of equal toxicity to Pba as sulphuric acid. In contrast, carfentrazone appeared to be non-toxic to the bacterium. These results may be useful in interpreting the results of field trials conducted under project 114R475 which aim to investigate the effects of different haulm desiccants on blackleg incidence and progeny tuber contamination under field conditions.

6. CONCLUSIONS

- *Pectobacterium atrosepticum* from seed of UK origin remains the most prevalent cause of blackleg disease, found to occur in some 23% of the total number of seed stocks entered for classification in England and Wales.
- *Pectobacterium carotovorum subsp. brasiliensis, Pectobacterium wasabiae* and a subgroup of *P. carotovorum subsp. carotovorum* were also confirmed for the first time in England and Wales in around 5-12% of blackleg cases in seed potato stocks entered for classification in England and Wales.
- *Pectobacterium carotovorum* subsp. *brasiliensis* appears to be newly emerging whereas *P. wasabiae* and the blackleg causing sub-group of *P. carotovorum* subsp. *carotovorum* appear to have been distributed in seed produced in GB and elsewhere around Europe for many years.
- *P. carotovorum* subsp. *brasiliensis* was mostly found in stocks grown from seed imported directly from the Netherlands, although it was also found for the first time in 2015 in stocks grown from seed of GB origin.
- *Dickeya solani* and *Dickeya dianthicola* remain minor causes of blackleg in seed potato stocks entered for classification in England and Wales, although *D. dianthicola* was also detected for the first time in 2015 in a seed potato stock of GB origin.
- A new variable number tandem repeat (VNTR) DNA typing scheme was developed which distinguishes at least 18 identifiable strains of *Pectobacterium atrosepticum*. This has been used to further investigate sources and pathways of contamination during the first year field generation of mini-tuber to PB-1 seed crops produced in both Scotland and England.
- Contamination of progeny tubers often occurs during the first field generation from mini-tubers with *Pba* isolates originating from the local environment.
- Sources of contamination during the first generation from mini-tubers most likely include blackleg plants from crops of lower grade growing in the vicinity.
- Planting mini-tubers in isolation from other seed and ware stocks may reduce risk of contamination with *Pba*.
- *Pba* contamination levels on seed tuber stocks increase with subsequent field generations.
- Additional sources of contamination, especially for field generations 2 and above, include the contaminated seed stock and contaminated machinery (including harvesters).

- A simplified method of testing potato tubers based on sampling stolon end cores rather than tuber peel gave reliable and comparable results to currently used methods and could be used to reduce cost and increase efficiency of post-harvest tuber testing.
- A simple *in vitro* test showed that commercial haulm dessicant formulations of sulphuric acid and diquat were equally toxic to *Pectobacterium atrosepticum*, whereas carfentrazone was non-toxic, even when exposed to the undiluted formulation.

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