Relative Incidence of *Erwinia carotovora* subsp. *atroseptica* in Stolon End and Peridermal Tissue of Potato Tubers in Canada

S. H. De Boer, Center for Animal and Plant Health, Canadian Food Inspection Agency, 93 Mount Edward Road, Charlottetown, PE C1A 5T1, Canada

ABSTRACT

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An enrichment enzyme-linked immunosorbent assay (ELISA) using monoclonal antibody 4F6, which is specific for the lipopolysaccharide antigen of serogroup I strains of *Erwinia carotovora* subsp. *atroseptica*, was used to detect the blackleg pathogen in potato seed lots after having confirmed that this serogroup is still the predominant strain of the pathogen in Canada. *E. carotovora* subsp. *atroseptica* was detected on <6% of tubers in most seed lots, but the level of tuber contamination increased with the number of field generations. The incidence of *E. carotovora* subsp. *atroseptica* was generally greater in tissue taken from the stolon end of tubers than from peel samples, although there was a positive correlation between the bacterium's presence in the two sample types. The incidence of *E. carotovora* subsp. *atroseptica* in seed tuber lots was estimated from the results of tests on multiple composite samples, providing a cost effective approach for indexing seed lots for blackleg risk. The incidence of *E. carotovora* subsp. *atroseptica* was two and three times greater for both stolon end and peel samples, respectively, after harvest and storage of seed lots compared with tubers hand dug just before commercial harvesting, which suggests that the process whereby tubers become contaminated has both field and postharvest components.

Additional keywords: indexing, PCR, soft rot

The blackleg disease of potato is caused by the bacterium *Erwinia carotovora* subsp. *atroseptica*. Although it has been reported that blackleg-like symptoms also may be caused by *E. carotovora* subsp. *carotovora* and *Erwinia chrysanthemi* (21,25), there is no evidence that these bacteria play a role in the epidemiology of blackleg in Canada.

Potato seed tubers are the chief source of inoculum for the blackleg disease. The work of Perombelon (26), in particular, has shown that *E. carotovora* subsp. *atroseptica* in seed tubers incites decay of the tuber after planting, and subsequent movement of the bacterium throughout the root zone of the plant results in contamination of progeny tubers, completing the cycle. Tuber lenticels are a particularly favorable niche for survival of the bacterium (1). However, the relationship between incidence and extent of tuber contamination and blackleg expression under field conditions not only differs among cultivars but also is affected by growing conditions (2).

In various aspects, the blackleg disease is similar to the other two major bacterial

diseases of potato, namely bacterial ring rot and brown rot caused by *Clavibacter michiganensis* subsp. *sepedonicus* and *Ralstonia solanacearum*, respectively. For both ring rot and brown rot, the seed tuber is also the major inoculum source (8,11). These bacteria are known to be present in the vascular tissue, from which they move to the developing plant and hence to the progeny tubers via the stolons. There has been little emphasis in blackleg studies on the presence of *E. carotovora* subsp. *atroseptica* in vascular tissue. Recently, however, Helias et al. (15) examined stolon tissue on plants grown from *E. carotovora* subsp. *atroseptica*-inoculated tubers and found the bacterium in almost all stolon samples and suggested that stolons were also an important contamination pathway. Perhaps *E. carotovora* subsp. *atroseptica*, like *C. michiganensis* subsp. *sepedonicus* and *R. solanacearum*, also has a significant vascular presence.

Since planting of pathogen-free seed tubers is an important control strategy for bacterial diseases of potato, there has been much emphasis on tuber indexing methods to differentiate among infected and noninfected potato seed lots. Indexing methodologies for ring rot and brown rot have almost exclusively focused on detection of the bacterium at the stolon attachment site of the tubers (8,11,17). In contrast, indexing methodologies for blackleg have very much emphasized the potato periderm or peel as the site of bacterial inoculum (3,13,16,18,34). Although the stolon end

has not been entirely ignored as a site of contamination with *E. carotovora* subsp. *atroseptica*, it has not been specifically targeted for testing (29).

The possibility that there are two more or less independent populations of *E. carotovora* subsp. *atroseptica* associated with tubers must be considered—one population emanating from decaying seed pieces and causing external contamination of progeny tubers, and the other population originating from the vascular tissue of plants and migrating through the vascular tissue of stolons into the tuber. Even if the two populations are not strictly independent, their epidemiological importance may differ. In this study, we specifically evaluated incidence of *E. carotovora* subsp. *atroseptica* in the stolon end of tubers and compared this incidence with that detected in tuber peel.

MATERIALS AND METHODS

Tuber samples. Seed potato tubers from the provinces of Alberta, New Brunswick, and Prince Edward Island were tested for the presence of *E. carotovora* subsp. *atroseptica* either individually or as composite samples. For composite samples, the tissue from either 10 or 200 tubers was combined into a single sample.

Tubers, washed in running tap water, were sampled by removing a 0.5-g cone-shaped plug of tissue from around the stolon attachment site with a scalpel. Alternatively, a strip of 2-mm-thick peel tissue about 2 cm wide and 6 to 9 cm long, depending on the size of the tuber, was removed with a kitchen potato peeler. For experiments in which stolon ends and peel samples were compared, the same tubers were used for both samples. In other experiments, only stolon ends were sampled. Single stolon end samples or composites from 10 tubers were placed in plastic bags with equal amounts (vol/wt) of water and macerated by tapping with a mallet; composites of 200 stolon ends were macerated in a blender with equal amounts of water (vol/wt). Peel samples were crushed with a Pollähne press (MEKU, Wennigsen, Germany) and the sap collected in tubes for individual samples or combined for composite samples. The liquid phase from each of the different macerates was used for testing.

Isolation and characterization of *E. carotovora* subsp. *atroseptica*. Samples were streaked on crystal violet pectate (CVP) medium (4) prepared with Sunkist polypectate (no longer commercially avail-

Corresponding author: S. H. De Boer
E-mail: deboers@inspection.gc.ca

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able) and incubated at 15°C for 3 to 4 days. Characteristic erwinia-like colonies were picked from the CVP medium and cultured from single colonies on nutrient agar until pure cultures were attained. Isolates were tested for growth on α -methylglucoside, production of reducing substances from sucrose, growth at 37°C, and phosphatase activity as described (6).

Enrichment. Samples were enriched for *E. carotovora* subsp. *atroseptica* by adding 100 μ l of sample to 1 ml of polypectate enrichment medium (6) and incubating at 15°C for 3 to 4 days.

Enzyme-linked immunosorbent assay (ELISA). Samples were tested by ELISA both before and after enrichment. An indirect sandwich ELISA procedure was used with monoclonal antibody 4F6, specific for serogroup I of *E. carotovora* subsp. *atroseptica* (7). The procedure was modified as follows. Fifty μ l of sample buffer (0.05 M phosphate buffer pH 7.2, 0.85% NaCl, 2% polyvinylpyrrolidone, 0.2% skim milk powder, 0.05% Tween 80) was loaded in wells of polystyrene plates previously coated with trapping polyclonal antibody prior to loading the sample. Samples were incubated overnight at 4°C, but the monoclonal antibody and alkaline phosphatase-antibody conjugate were each incubated for 1 h at 37°C. The *p*-nitrophenyl-phosphate substrate was incubated for 1 h at room temperature before reading absorbance at 405 nm. Each plate was loaded with two positive and two negative control samples consisting of lyophilized bacterial supernatant fluid adjusted to give a constant absorbance reading and healthy potato extract, respectively. Sample absorbance values were transformed by $Y_t = (Y_x - Y_n)/(Y_p - Y_n)$, where Y_t is the transformed value, Y_x the absorbance reading, Y_n the mean of the absorbance of the negative controls, and Y_p the mean absorbance of the positive controls. Y_t values greater than or equal to 0.06 were considered positive.

Polymerase chain reaction (PCR). PCR amplification was done on a limited number of samples using primers ECA1f and ECA1r as described (10).

Estimation of incidence. To determine the incidence of *E. carotovora* subsp. *atroseptica* in a potato lot, 40 composite samples of 10 tubers were tested. The incidence, as the percentage of contaminated or infected tubers in a seed lot, was estimated from the probability of detecting one or more positive tubers in a composite sample. The incidence (I) was calculated by $I = \{1 - [(N - p)/N]^{1/n}\} 100$, where p is the number of composites that tested positive for *E. carotovora* subsp. *atroseptica*, N the total number of composites tested, and n the number of tubers combined into a composite sample (23).

RESULTS

Preliminary experiments. To confirm that the blackleg disease in Canada was

still chiefly caused by serogroup I of *E. carotovora* subsp. *atroseptica*, as was determined previously (9), 71 potato stems with blackleg-like symptoms representing 19 different seed potato lots were tested for reaction with monoclonal antibody 4F6 in ELISA. Of these, 91.5% tested positive.

Since ELISA was the method of choice for testing large numbers of seed potato lots in a cost-effective manner, adequacy of ELISA was investigated by applying confirmatory tests on preliminary samples. The presence of *E. carotovora* subsp. *atroseptica* was confirmed in more than 70% of the samples that were positive in ELISA with and without prior enrichment by isolation of *E. carotovora* subsp. *atroseptica* on CVP medium (Table 1). About 80% of ELISA-positive samples could be confirmed by PCR amplification of a 690-bp DNA fragment using *E. carotovora* subsp. *atroseptica*-specific primers (Table 1).

ELISA with and without enrichment. Extracts from many of the seed potato lots in this study were tested by ELISA both with and without prior enrichment. Detection limits of ELISA and enrichment-ELISA are known to be in the range of 10^5 to 10^6 cells per ml and 10^2 to 10^3 cells per ml, respectively (19). As expected, with the prior enrichment, *E. carotovora* subsp. *atroseptica* was found in more samples than without enrichment. In four sets of seed lots, *E. carotovora* subsp. *atroseptica* was detected in two to three times as many lots with prior enrichment compared with the number found without enrichment (Table 2).

Detection of *E. carotovora* subsp. *atroseptica* in stolon samples from seed

tubers. In 1994, 400 tubers from each of 886 seed lots grown in New Brunswick were tested as two composite samples of stolon end tissue from 200 tubers by ELISA without enrichment. The samples were collected and processed for regulatory bacterial ring rot testing according to a standard protocol (8), and the homogenate was tested in ELISA for the presence of *E. carotovora* subsp. *atroseptica* in addition to the test for *C. michiganensis* subsp. *sepedonicus*. Seed lots were grouped according to certification class, which generally represents the number of field generations of the seed lot. The number of lots positive for *E. carotovora* subsp. *atroseptica* varied from 9% for seed in the Elite I class (second field generation) to 30% for the Certified class (seventh field generation) (Fig. 1). For the remaining seed lots, the level of positive reactions varied from 13 to 18%. There was a significant correlation ($R^2 = 0.722$) between the number of seed lots with *E. carotovora* subsp. *atroseptica* and number of field generations.

Relative incidence of *E. carotovora* subsp. *atroseptica* in stolon and peel samples. The incidence of *E. carotovora* subsp. *atroseptica* in stolon end and peel samples within seed lots was assessed during 1998 and 1999 using 12 seed lots from Alberta and 50 lots from Prince Edward Island (PEI). For each seed lot, 400 tubers were tested by enrichment ELISA in composites of 10 stolon end and 10 peel samples. The incidence in a seed lot was estimated on the basis of the number of positive composite stolon end and peel samples (Table 3). *E. carotovora* subsp. *atroseptica* was not detected in either peel

Table 1. Confirmatory tests to substantiate results from enzyme-linked immunosorbent assay (ELISA) for the detection of *Erwinia carotovora* subsp. *atroseptica* in potato tissue

ELISA method	Confirmation test method ^a	Samples (no.)	Percent confirmed positive
Without enrichment	Isolation on CVP	17	76.5
	PCR amplification	11	81.8
With enrichment	Isolation on CVP	49	71.4
	PCR amplification	39	79.5

^a CVP = crystal violet pectate; PCR = polymerase chain reaction.

Table 2. Comparison of number of seed lots found to contain *Erwinia carotovora* subsp. *atroseptica* by enzyme-linked immunosorbent assay (ELISA) with and without prior enrichment of tissue extracts

Set of seed lots	Seed lots in set (no.)	Positive seed lots (no.) ^a			
		Non-enriched-ELISA		Enriched-ELISA	
		Peel tissue	Stolon end tissue	Peel tissue	Stolon end tissue
A	12	4	5	6	7
B	23	0	2	5	12
C	23	6	8	14	15
D	50	6	11	19	25
Total	108	16	26	44	57

^a Number of seed lots in which *E. carotovora* subsp. *atroseptica* serogroup I was detected by ELISA in tissue samples taken from the stolon end and peel of the same tubers.

or stolon end samples in 8% of the Alberta seed lots and 18% of the seed lots from PEI. Incidence of *E. carotovora* subsp. *atroseptica* was generally low in lots in which it was found; the highest incidence being 30% in an Elite 3 seed lot from PEI (Table 3). There were 11 seed lots in which it was found only in the stolon end samples but not in the peel samples, while there were only three lots in which it was found in the peel samples but not in the stolon end samples. Nevertheless, there was significant correlation ($R^2 = 0.797$) between the overall incidence in stolon end and peel samples of a given tuber lot (Fig. 2).

Impact of harvesting on *E. carotovora* subsp. *atroseptica* contamination. It has been suggested that potato tubers may acquire *E. carotovora* subsp. *atroseptica* contamination postharvest through the harvesting and grading procedures in addition to the field acquisition of the bacterium (12). Hence, we tested tubers before normal harvest by hand digging tubers

directly from the field and resampling the same lots after they had been harvested, graded, and placed in storage. Both pre- and postharvest samples were stored at 4°C for 5 to 6 months before testing by enrichment-ELISA. The frequency with which *E. carotovora* subsp. *atroseptica* was detected was higher for the postharvest samples for both peel and stolon end samples compared with preharvest values (Table 4). Incidence of *E. carotovora* subsp. *atroseptica* differed among cultivars with Sebago, a cultivar known to be particularly susceptible to blackleg, having a higher incidence of *E. carotovora* subsp. *atroseptica* than other cultivars. Overall the incidence in peel samples was threefold greater for postharvest than for preharvest samples, and the difference was twofold for stolon end samples (Table 4).

DISCUSSION

In 1987, 96% of isolates of *E. carotovora* subsp. *atroseptica* from blackleg-

infected plants in Canada were serogroup I (9). Serogroup I has also been the predominant type in several other countries. Recently, however, it was reported in Poland (31) that the proportion of serogroup I strains in blackleg-infected plants was lower, at about 65%, and this is perhaps also true elsewhere. Our results indicate that serogroup I was still the predominant *E. carotovora* subsp. *atroseptica* type associated with blackleg in Canada. In Europe, some of the blackleg-like symptoms are caused by *E. chrysanthemi* (32). Although *E. chrysanthemi* has never been associated with potato in Canada, we tested 50 seed lots in ELISA with monoclonal antibody 6A6 (30) specific for *E. chrysanthemi* but found no positives (unpublished data), confirming that this species is not associated with potato in Canada.

Efficacy of ELISA for detecting *E. carotovora* subsp. *atroseptica* in potato tubers depends on the serological specificity of the antibody and the sensitivity of the test. Monoclonal antibodies to the lipopolysaccharide of *E. carotovora* subsp. *atroseptica* are highly specific but may cross-react with a few other bacteria common in the soil environment (33). While it is doubtful that these other bacteria achieve adequate populations to be detectable by ELISA, there is a possibility that false positive ELISAs could occur. The results of our confirmatory tests suggest that ELISA is reasonably accurate in detecting true *E. carotovora* subsp. *atroseptica*-positive samples (Table 1). It must be kept in mind, also, that the confirmatory tests themselves have their difficulties. The isolation procedure on the CVP semiselective medium may be limited by overgrowth or inhibition by saprophytic bacteria that inevitably compete with the target bacterium. PCR amplification, too, is prone to give false negative results due to inhibitory substances in sample extracts.

The limitation in sensitivity of the ELISA is overcome to a great extent by using enrichment-ELISA (19). This ap-

Fig. 1. Percentage of lots in different classes of seed potatoes grown in New Brunswick in which *Erwinia carotovora* subsp. *atroseptica* serogroup I was detected by enzyme-linked immunosorbent assay (ELISA). Tests were conducted on two composite samples per seed lot, each containing 200 stolon ends. Seed lots included 52 cultivars, and number of lots per seed class tested were 22 for Elite 1, 105 for Elite 2, 288 for Elite 3, 281 for Elite 4, 147 for Foundation, and 43 for Certified.

Table 3. Incidence of *Erwinia carotovora* subsp. *atroseptica* in the periderm and stolon end of tubers in potato lots of different seed classes from Alberta and Prince Edward Island

Seed class	Total lots	Incidence (%) of <i>E. carotovora</i> subsp. <i>atroseptica</i> ^a					
		Peel tissue			Stolon end tissue		
		Contaminated lots	Mean incidence	Incidence among positives	Contaminated lots	Mean incidence	Incidence among positives
Alberta							
Pre-Elite	1	0	0	na	0	0	na
Elite 1	3	1	0.13	0.4	3	0.37	0.3-0.4
Elite 2	4	3	0.25	0.3-0.4	2	0.28	0.3-0.8
Elite 3	4	2	0.75	0.5-2.5	2	1.18	2.2-2.5
Prince Edward Island							
Elite 1	9	1	0.14	1.24	6	0.28	0.25-0.78
Elite 2	9	3	0.73	0.25-5.70	3	1.03	1.91-4.22
Elite 3	14	10	5.97	0.25-30.12	11	3.68	0.25-16.40
Elite 4	10	7	5.55	0.51-17.28	10	5.43	0.25-18.77
Foundation	8	7	3.56	0.53-16.00	8	1.88	0.25-5.38

^a Percentage of tubers within a seed lot that were contaminated with *E. carotovora* subsp. *atroseptica* serogroup I as estimated from the number of composite samples out of 40 that were positive by enrichment enzyme-linked immunosorbent assay (ELISA).

proach has been quite successful, not only for pectolytic erwinias, but also for other pathogenic bacteria for which there are good semiselective enrichment media (20). We found *E. carotovora* subsp. *atroseptica* in two to three times as many seed lots using enrichment-ELISA than using ELISA on the raw extract without enrichment (Table 2).

Notwithstanding its limitations, enrichment-ELISA could be used to provide a relative estimation of the incidence of *E. carotovora* subsp. *atroseptica* in seed lots. *E. carotovora* subsp. *atroseptica* was detected in only about half of the tuber lots examined (Table 2), and when it was detected, the incidence was very low (Table 3). The low incidence at which *E. carotovora* subsp. *atroseptica* was detected is consistent with the low level of blackleg that occurs in potato in Canada. Previously, the incidence of blackleg was greatly diminished by the use of pathogen-tested stem cuttings to initiate the nuclear level of seed potatoes (5). Today, stem cuttings have been replaced by pathogen-tested micropropagated plantlets and minitubers to initiate seed tuber lots. This appears to have been an effective approach in further reducing the occurrence of blackleg and is similar to the decrease in incidence of blackleg in other geographic areas where pathogen-tested propagules have been used (27). The observation that most seed potato tubers are contaminated with *E. carotovora* subsp. *atroseptica* (26) is not true of Canadian seed potatoes today.

The reduction in blackleg by the use of *E. carotovora* subsp. *atroseptica*-free planting material is consistent with the notion that the primary inoculum source is the seed tuber. The disease can be controlled by eliminating the seed tuber as an inoculum source as is done for the bacterial ring rot (8) and brown rot diseases (11). Disease reduction, however, has not translated into disease eradication. *E. carotovora* subsp. *atroseptica*-free material set out in the field sometimes becomes contaminated, as evidenced by the detection of *E. carotovora* subsp. *atroseptica* on seed lots in this study and the occasional find of blackleg in the field. The presence of *E. carotovora* subsp. *atroseptica* on Elite 1 seed lots (Fig. 1) representing the second field generation suggests that contamination can occur early on in the field environment. The number of seed lots contaminated increased with the number of generations in the field (Fig. 1).

The initial source of *E. carotovora* subsp. *atroseptica* that contaminates or infects clean stocks is unclear. Reports in the literature had suggested that surface contamination of tubers may result from contact with inoculum on harvesters or grading lines contaminated from as little as a single infected tuber (12). In our study, we indeed found almost threefold higher levels of *E. carotovora* subsp. *atroseptica*

contamination in potatoes that had been harvested, graded, and stored by normal agronomic practices compared with tubers hand dug from the field (Table 4). However, we also found a twofold increase in the incidence of *E. carotovora* subsp. *atroseptica* in stolon end samples in normally harvested tubers compared with preharvest hand dug tubers. This latter increase is more difficult to explain by mere contact with an inoculum source. Perhaps this increase was due to late-season movement or multiplication of bacteria in the stolons. In any case, the source of the initial inoculum needs to be investigated. Although *E. carotovora* subsp. *carotovora* is known to survive in the soil environment and in surface water, *E. carotovora* subsp. *atroseptica* is seldom encountered in studies of pectolytic erwinia in the environment (14,22,24).

The incidence of *E. carotovora* subsp. *atroseptica* in stolon end samples tended to be higher than the incidence in peel samples. For the bacterial ring rot and brown

rot pathogens, it is assumed that they reside in the stolon end of tubers, having migrated from the plant stems via the stolons, but whether this is always true of *E. carotovora* subsp. *atroseptica* has not been ascertained. The finding of the pathogen in stolons by Helias et al. (15) adds credence to the possibility that indeed it is transmitted to tubers in this manner, even in the absence of overt symptoms of disease. Stolon-transmitted bacteria would necessarily be associated with the vascular tissue of the tubers. Perhaps such vascular-associated bacterial populations can travel to developing stems when the tubers are used for seed and cause blackleg disease more readily than populations harbored in the lenticels and other protected niches on the tuber surface.

The populations of *E. carotovora* subsp. *atroseptica* in stolon ends of tubers has significant implications for strategies to index seed tuber lots for the presence of the bacteria. Methodologies to index for ring rot and brown rot by sampling tuber

Fig. 2. Comparison of the incidence of *Erwinia carotovora* subsp. *atroseptica* in stolon samples with the incidence in peel samples for seed lots from Prince Edward Island tested by the enrichment enzyme-linked immunosorbent assay (ELISA) procedure.

Table 4. Frequency of *Erwinia carotovora* subsp. *atroseptica* in potato peel and stolon end samples for tubers collected prior to and after harvest

Cultivar	Composites ^b tested	Positive composite samples (no.) ^a			
		Peel samples		Stolon end samples	
		Preharvest	Postharvest	Preharvest	Postharvest
Russet Burbank	160	0	1	14	15
Russet Norkota	160	0	2	4	12
Sebago	120	14	32	12	22
Shepody	80	0	3	0	4
Superior	120	1	7	0	6
Other	240	10	28	12	18
Total	880	25	73	42	77

^a Number of composite samples of tuber stolon ends and peels in which *E. carotovora* subsp. *atroseptica* serogroup I was detected by enrichment enzyme-linked immunosorbent assay (ELISA).

^b Composites were of 10 tubers each.

stolon ends have been well developed and used extensively (8,11). This approach could be used equally well for detecting *E. carotovora* subsp. *atroseptica* in stolon ends. One important difference is that *E. carotovora* subsp. *atroseptica* does not have a zero tolerance like ring rot and brown rot. Hence, it is not enough to simply detect the pathogen; there is a need to establish its incidence when present. Testing large numbers of tubers individually would be prohibitively costly, and testing in composites of 200 tubers as for ring rot and brown rot would not provide incidence levels. It may, however, be feasible to use the approach taken in this study. Per seed lot, we tested 40 composite samples of 10 tubers each. For independent random samples, the incidence in a lot can be estimated from the number of positive composite samples if the incidence is low enough (<30% if 40 composites are used) to avoid multiple positive tubers per composite sample. This approach with the ELISA procedure is feasible and of reasonable cost. In geographic areas where multiple or unknown serotypes of *E. carotovora* subsp. *atroseptica* occur, other testing strategies may need to be used, such as PCR testing (13) or isolation procedures (28).

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