Oxygen Status of Potato Tuber Tissue in Relation to Maceration by Pectic Enzymes of *Erwinia carotovora*

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ABSTRACT

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Tissue maceration in tubers injected with filter-sterilized culture filtrate containing pectolytic enzymes from *Erwinia carotovora* (Ec) was compared to that produced by injections of viable suspensions of Ec at low and ambient oxygen levels. Both types of injection resulted in rapid decay of tuber tissue under low oxygen conditions; in contrast, no decay occurred in tissue incubated in air. Lesions formed in enzyme-injected anaerobic tubers were similar to those induced by inoculation with cells of Ec. Absence of maceration in air was not attributable to loss of enzyme activity at the injection site. Pectate lyase (PL) activity declined almost fivefold when culture filtrates were stored aerobically for 96 hr; however, macerating activity was still present. Furthermore, cultures of Ec grew more rapidly and reached higher population levels when cultured aerobically in vitro than under anaerobic conditions. Under aerobic conditions a host-mediated response affecting pectic enzyme substrates and/or activity may contribute to the initial resistance of potato tubers to bacterial soft rot infection before direct inhibition of bacterial growth occurs.

Additional key words: proteolytic enzymes, *Solanum tuberosum*.

Bacterial soft rot caused by *Erwinia carotovora* is more severe when infected potato tubers are held under conditions of reduced oxygen (14,16,17,19). Low oxygen tensions develop in storage when potatoes are covered with a film of water from condensation or in transit when washed potatoes are not dried before packaging. When a water film covers tuber surfaces, oxygen levels decline to zero in 2.5 hr at 21 C (4). The number of colony-forming units (CFU) of *E. carotovora* required to elicit a soft rot lesion in potato tubers declines from 10^9 to 10^5 CFU per injection site as oxygen concentration decreases from 20 to 5% (7). On the basis of these and related studies, tests to determine bacterial soft rot potential of tubers are usually conducted under conditions of reduced oxygen (3,7,9,15). The increased resistance to decay as oxygen levels increase may reflect either physiological changes in tuber tissue altering susceptibility to maceration (1,14,27) or direct inhibition of bacterial growth (12,14,17,23). In previous studies on the mechanisms involved in tissue maceration by pectic enzymes, observation of possible effects of oxygen on the process were not reported.

The objective of this study was to examine the relationship of oxygen status to maceration of potato tuber tissue by pectolytic enzymes of *E. carotovora*.

MATERIALS AND METHODS

Bacterial strains and media. *Erwinia carotovora* pv. *atroseptica* (van Hall) Dye (isolate SR 8) and *E. carotovora* pv. *carotovora* (Jones) Bergey et al (isolates SR 16, SR 318, and SR 319) were used in this study. (These names will be abbreviated Eca and Ecc in this article.) SR8 and SR 16 were originally isolated from potato tubers, SR 318 was isolated from a potato stem, and SR 319 from potato field soil in Wisconsin. SR 8 reacts positively with serogroup I antiserum, SR 318 with serogroup V, and SR 319 with serogroup XXIX (6). Cultures were maintained as suspensions in distilled water and grown on a casamino acids-peptone-glucose (CPG) agar medium (5). Cultures of Eca or Ecc that had been incubated at 22 C for 48–72 hr were transferred from CPG plates to 1-L flasks containing 500 ml of pectate salts medium ($MgSO_4$, 0.02%; KH_2PO_4, 0.15%; Na_2HPO_4, 0.715%; $[NH_4]_2SO_4$, 0.30%; sodium polypectate [NaPP, R. L. Kluft and Co., Ltd., Oconomowoc, WI 53066], 1.00%), pH 7.0. Flasks were incubated at 24 C on a rotary shaker for 4 days.

Growth curves. Side-arm flasks (250 ml) containing 100 ml CPG broth were inoculated with 1.0 ml of 48-hr cultures of *E. carotovora* (strains SR 8, SR 316, SR 318, SR 319) adjusted to an $A_{620\ nm}$ of 0.13, and incubated at 24 C on a rotary shaker at 128 rpm for 22 hr. To maintain low oxygen cultures, freshly autoclaved and cooled CPG medium in rubber-stoppered flasks was sparged with nitrogen gas for several minutes before and after inoculation. Aerobic flasks had cotton stoppers. Turbidity was recorded using a Klett-Summerson colorimeter at intervals of 1–2 hr.

Enzyme preparation. Following centrifugation at 12,000 *g* for 20 min, the supernatant fluid from 4-day-old cultures of *Erwinia* in pectate salts medium was decanted and ammonium sulphate was added to bring the solutions to 40, 60, 80, and 90% saturation. The solutions were stirred at 4 C for 1 hr or more, then centrifuged as before. The precipitate formed in each fraction was resuspended in 3–5 ml of distilled H_2O and dialyzed overnight at 4 C against distilled H_2O. Dialyzed preparations were stored at −10 C. Protein concentrations were determined by using the Lowry method (13).

Enzyme assays. Pectolytic enzyme activity for strains SR 8, SR 318, and SR 319 was determined by rotating spindle viscometry (20) and by spectrophotometric methods. Samples of culture filtrates were heat-treated in a boiling water bath for 15 min, cooled, then substituted for untreated culture filtrate in enzyme reaction mixtures as controls.

The viscometric reaction mixture for the pectate lyase (PL) assay was 2 ml of 2.0% NaPP in 0.05 M tris-HCl buffer (pH 8.5), 0.5 ml of 0.05 M tris-HCl buffer (pH 8.5), and 0.5 ml culture filtrate. One milliliter of reaction mixture was added to the chamber of a Brookfield rotating-spindle viscometer (Brookfield Engineering Laboratories, Stoughton, MA 02072) at 24 C and readings were taken at 1 min, then every 2–5 min thereafter for 20–30 min. PL activity in culture filtrates was confirmed by recording the increase in absorbance at 230 nm (22) in a reaction mixture containing 1 ml

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of 0.2% NaPP in 0.2 M tris-HCl buffer (pH 8.0), 1.0 ml of distilled water, and 1.0 ml of culture filtrate enzyme preparation. Reference blanks were prepared using water or heat-treated culture filtrate.

Polygalacturonase (PG) activity was measured by the viscometric technique described above, substituting 0.05 M sodium acetate buffer (pH 4.5) for the tris buffer, and by testing levels of reducing sugars (18) in a reaction mixture containing 1.0 ml of 0.2% NaPP in 0.2 M sodium acetate buffer (pH 4.5), 1.0 ml of water, and 1.0 ml of culture filtrate.

Proteolytic enzymes were assayed according to the technique outlined by Tseng and Mount (24). The proteolytic enzyme assay reaction mixture contained 1.0 ml of 1.0% gelatin and 0.01% $CaCl_2$ in 0.05 M tris-HCl buffer (pH 8.0) and 1.0 ml of the culture filtrate. After 1 hr at 24 C, 3.0 ml of 20% trichloroacetic acid (TCA) was added and mixed; then the preparation was centrifuged at 3,000 *g* in a Sorvall SS34 rotor for 20 min at 5 C. The absorbance of the supernatant was determined at 280 nm on a Varian DB spectrophotometer standardized with a reference tube prepared by adding 3.0 ml of 20% TCA to a reaction mixture immediately after addition of enzyme sample.

Injection technique. Potato tubers (cultivar Russet Burbank) that had been stored at 4 C since harvest were immersed twice in 0.05% sodium hypochlorite solution for 20-min periods, rinsed with distilled water, and allowed to air dry. Sterilized micropipet tips (Pipetman, Rainin Instrument Co., Woburn, MA 01801) containing injection solutions were inserted into the potato to a depth of 15 mm and left in place. Injection volumes were 0.1 and 1.0 ml. Injected potatoes were incubated in anaerobe jars with palladium catalyst (Baltimore Biological Laboratories, Cockeysville, MD 21030) and 80% (v/v) nitrogen, 10% (v/v) hydrogen, and 10% (v/v) carbon dioxide. All incubations were at 20 C. In each test, one series was incubated under aerobic conditions. Prior to injection, culture filtrates were filter-sterilized by passage through 0.45-μm (pore size) Millipore filters. Most of the macerating activity was observed in the resuspended precipitate from the 90% $(NH_4)_2SO_4$ fraction; thus, this fraction was used routinely in injections. Following incubation, injection sites were tested for sterility by streaking from each lesion on two CPG agar plates, then incubating one anaerobically and the other aerobically.

Tuber extractions. To determine whether pectic enzyme activity could be recovered from potato tissue following aerobic and anaerobic incubation for 4–5 days, tuber tissue was excised around the injection site, transferred to glass vials, and immediately frozen in an acetone-dry ice bath. Tissue samples were thawed, then crushed in distilled water by using a glass rod, and these preparations were centrifuged at 10,000 *g* for 15 min. The supernatant fluids were dialyzed overnight against distilled water, filter-sterilized, and stored frozen.

RESULTS

Growth under low oxygen conditions in vitro. Growth of Eca and Ecc under reduced oxygen was significantly less than in aerated cultures (Fig. 1). Both growth rate and yield were affected.

Enzyme production. Filtrates from cultures of *Erwinia* had heat-sensitive pectolytic enzyme activity at pH 8.5 and 4.5 (Fig. 2) corresponding to PL and PG activity, respectively. Results obtained in viscometric measurements were confirmed by spectrophotometric methods.

In vivo tissue maceration. When tubers were injected with either viable suspensions of Ecc or with a sterile culture filtrate and maintained in low-oxygen atmospheres, tissue was macerated at the injection points (Table 1, Fig. 3). In contrast, under aerobic conditions, a dry, necrotic lesion similar to that caused by simple

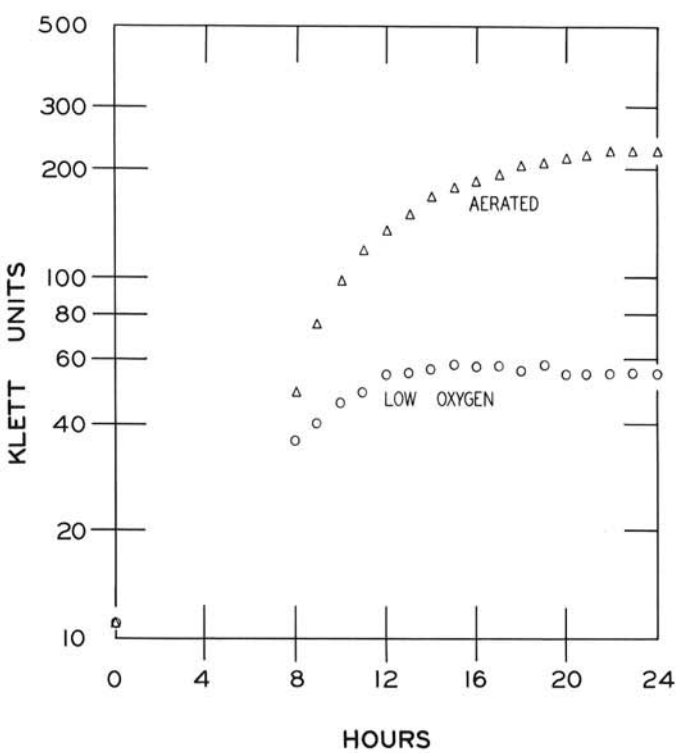

Fig. 1. Growth of *Erwinia carotovora* pv. *carotovora* (SR 319) in aerated (Δ) and low oxygen (o) broth cultures. Turbidity measurements made with a Klett-Summerson photoelectric colorimeter. Aerated culture grown in 100 ml of CPG broth in 250-ml flasks (with cotton plugs) for 22 hr at 25 C on a rotary shaker; in low-oxygen flasks, the broth was sparged with nitrogen after inoculation and the flasks were sealed with rubber stoppers.

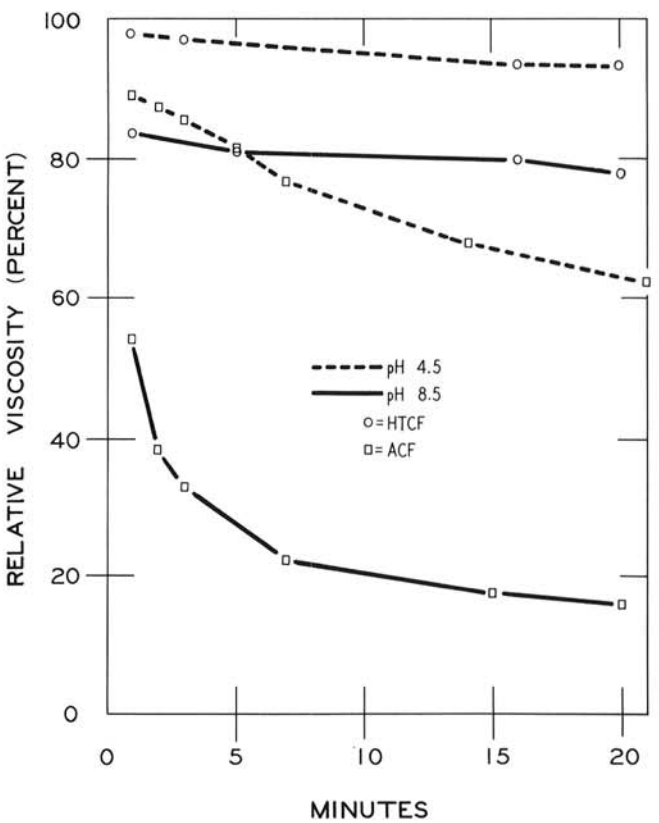

Fig. 2. Pectate lyase (PL) and polygalacturonase (PG) activity assayed viscometrically in active culture filtrate (ACF) from 4-day-old pectate-salts culture of *Erwinia carotovora* pv. *carotovora* (SR 319). Reaction mixture for PL activity (pH 8.5) (——) was 2 ml of 2% NaPP in 0.05 M tris-HCl buffer (pH 8.5), 0.5 ml of 0.05 M tris-HCl buffer (pH 8.5), and 0.5 ml active culture filtrates (□); for PG activity (pH 4.5) (---) sodium acetate buffer (pH 4.5) was substituted for the tris buffer. Heat-treated culture filtrate (HTCF) was held in a boiling water bath for 15 min, then substituted for ACF in reaction mixtures (o). One milliliter of reaction mixture was added to Brookfield rotating spindle viscometer at 24 C.

mechanical injury formed at the point at which either cells of Ecc or filter-sterilized culture filtrate were injected. Injection of a heat-treated culture filtrate caused no tissue maceration under aerobic or anaerobic conditions (Table 1). No bacterial growth was observed on either aerobically or anaerobically incubated CPG plates streaked from wound lesions 2, 4, and 7 days after injection with sterilized filtrate.

Reduced PL activity was recovered in aqueous extractions from potato sites injected prior to incubation with culture filtrate preparations containing high PL activity (Table 2). PL was present in extracts from tubers incubated under both anaerobic and aerobic conditions, although tissue maceration was induced only under low oxygen incubation.

Activity of culture filtrate in vitro. When a filter-sterilized culture filtrate of SR 319 was stored at ambient oxygen levels, PL activity declined from 146 to 31 μmoles of product formed per minute per milligram of protein in 96 hr. Tuber injections with this preparation induced tissue maceration under low-oxygen incubation, indicating that macerating activity of the preparation was not lost through aerobic exposure in vitro. In a separate experiment, PL activity in culture filtrates stored either aerobically or anaerobically declined 50% during 96 hr of incubation.

Proteolytic enzyme activity in culture filtrates. Although proteolytic enzyme activity was detected in culture filtrates and heat-treated filtrates used in potato injection tests, such activity could not be correlated with tissue degradation following incubation at either ambient or low oxygen levels (Table 3). The relative increase in proteolytic activity in the heat-treated sample may be due to a concentration effect resulting from a volume decrease during heat treatment.

DISCUSSION

As a facultative anaerobe, *E. carotovora* is able to grow when oxygen is limited, but our data and those of others (26) demonstrate that optimal growth occurs in vitro under aerobic rather than anaerobic conditions. In contrast, growth in vivo is more rapid when oxygen is limited (7).

The high resistance of potato tubers to bacterial soft rot in air has been attributed mainly to host response resulting in formation of compounds such as phytoalexins that directly inhibit bacterial growth (14). The data obtained in our studies using whole tubers injected with filter-sterilized pectolytic culture filtrates indicate that, in air, a host-mediated response to extracellular enzymes produced by the bacterium may be more important in resistance to maceration than direct inhibition of bacterial growth.

Macerating activity of pectolytic enzyme preparations has been studied using tuber disks or slices (2,8,12) rather than whole potato tubers. Since resistance to tissue maceration in whole tubers is oxygen-related when either viable Ec cells (7) or filter-sterilized pectic enzyme preparations are injected, the relevance of assays using tissue slices to in vivo conditions needs to be reevaluated. Since oxygen levels in tissue slice assays are not controlled, the

TABLE 1. Tissue maceration induced by cells and sterilized culture filtrates of *Erwinia carotovora* pv. *carotovora* SR 319 in potato tubers after 96 hr of incubation under aerobic and anaerobic conditions

Preparation injected[a]	Average wet weight of tissue macerated (g/injection site)[b]	
	Aerobic	Anaerobic
Cell suspension of *E. carotovora* (10^5 CFU/ml)	0	1.41 ± 0.36
Culture filtrate	0	0.15 ± 0.05
Heat-treated culture filtrate[c]	0	0

[a] Injection volumes were 0.1 ml.
[b] Means and standard deviation from three injection sites in three tubers.
[c] Culture filtrates were heat-treated in a water bath at 100 C for 15 min.

Fig. 3. Potato tubers sliced through the point of injection following incubation at 20 C for 96 hr under aerobic (left) and anaerobic (right) conditions. In both tubers, injections on the left side were 0.1 ml of a 10^7 cells per milliliter suspension of *Erwinia carotovora* pv. *carotovora* (Ecc); injections on the right side were 0.1 ml of the 90% ammonium sulphate fraction of a culture filtrate from a pectate-salts liquid media culture of Ecc that had been resuspended in water, dialyzed against water and filter-sterilized prior to injection. No bacterial contamination was noted in streak plates prepared from lesions at culture filtrate injection sites. Aerobic lesions are dry, dark, and firm; tissue maceration occurred only under anaerobic conditions and decayed tissue was removed and weighed.

TABLE 2. Tissue maceration and pectate lyase (PL) activity in aqueous extractions from potato tuber tissue following injection with culture filtrate from *Erwinia carotovora* pv. *carotovora* SR 319 and incubation at ambient or low oxygen levels for 96 hr at 20 C

Tubers injected[a] with	Type of incubation	Tissue maceration[b]	PL activity[c] in extract[d]
Culture filtrate[e]	Aerobic	0	73.5
	Anaerobic	0.33 ± 0.02	25.6
Heat-treated culture filtrate[f]	Aerobic	0	0
	Anaerobic	0	0

[a] Injection volumes were 1.0 ml.
[b] Average wet weight (g) of cream-colored decayed tissue per injection site.
[c] Amount (μmoles) of product formed per minute per milligram of protein as determined by increase in absorbance at 230 nm.
[d] Extracts prepared by combining 0.66–0.99 g of potato tissue from two injection sites from two potatoes per oxygen level.
[e] Crude culture filtrate (90% $(NH_4)_2SO_4$ fraction); PL activity in filtrate = 2,200 μmoles product formed per minute per milligram of protein.
[f] Same fraction as in footnote e after 15 min in a water bath at 100 C.

TABLE 3. The relationship of tissue maceration to pectate lyase (PL) and protease activity in a partially purified culture filtrate from *Erwinia carotovora* pv. *carotovora* SR 319[a]

	Culture filtrate	Heat-treated culture filtrate
PL activity[b] (μmoles product/min/mg protein)	494	0
Protease activity[c] (U/mg protein)	200	450
Tissue maceration[d] (Average g wet weight of tissue)	0.24	0

[a] Supernatant from pectate-salts liquid medium was saturated with 90% ammonium sulphate and centrifuged. The precipitate was resuspended in double distilled water and dialyzed against water overnight, then filter-sterilized by passage through 0.45-μm Millipore filters.
[b] PL activity was determined by increase in absorbance at 230 nm.
[c] Protease activity was determined in a reaction mixture containing 1.0 ml 1% gelatin, 0.01% $CaCl_2$ in 0.05 M tris-HCl buffer (pH 8.0) and 1 ml of culture filtrate.
[d] Tissue maceration was assessed in whole tubers following injection and low oxygen incubation. No maceration occurred following aerobic incubation.

water or buffer film in which experiments are conducted may reduce oxygen tensions to the degree that tissue becomes highly susceptible. Other factors, such as the thickness and size of tissue disks or slices, may also be involved.

Although PL in culture filtrates declined when stored aerobically in test tubes at room temperature, decreased activity in vitro does not explain the inability of the preparation to cause decay in aerobically incubated injected tubers. After 4 days of storage, a typical incubation period, these preparations were still capable of causing tissue maceration when injected into tubers and incubated anaerobically. In addition, in vitro incubation of culture filtrates at either oxygen level resulted in a gradual decline of enzymatic activity. These observations indicate that the failure of pectic enzymes to cause decay in injected tubers when incubated in air involves a reaction mediated by the host tissue rather than a direct effect of oxygen on the enzyme(s).

Proteolytic enzymes from *Pseudomonas* spp. macerate potato tuber tissue (25); however, heat-resistant proteases from Ecc are not responsible for tissue maceration in assays with partially purified culture filtrates. Furthermore, in injections with PD I (21), a purified pectic depolymerase kindly provided by Mark S. Mount, only those tubers incubated anaerobically decayed.

The results obtained with filter-sterilized pectolytic culture filtrates of *E. carotovora* in injections of whole tubers indicate that initial resistance to bacterial soft rot in air may not be attributed to phytoalexin production by the host tissue or to the inhibition of bacterial growth by toxic quinones formed by the oxidation of phenolic compounds. Phytoalexins and oxidized phenolics may have a direct inhibitory effect on the bacterium; however, since the resistant response is observed with partially purified extracellular pectolytic enzymes in the absence of the pathogen, a mechanism effectively limiting the action of pectic enzymes may be the primary mechanism of resistance.

The polymerization of proteins by quinones or free radicals (11) generated by oxidative enzymes from plant tissue may provide a mechanism for pectic enzyme inactivation in vivo under oxidative conditions. However, PL activity could still be recovered from extracts of injected tissues after aerobic incubation, although these tissues were not macerated. This indicates that if PL is bound by some plant product under ambient oxygen conditions, the binding may be reversible.

Resistance to bacterial soft rot under aerobic conditions can be explained on the basis that the pectate substrates in the middle lamella are protected in some manner from pectic enzymes produced by the bacterium. However, this resistant reaction may not be attributable to suberization and related wound healing responses (10), since these are reported to occur after localization of bacterial infection or limitation of tissue maceration by sterile pectolytic enzymes under ambient oxygen conditions in whole tubers. Time-course studies are needed to determine the specific nature of cell wall changes related to this possible mechanism of resistance to maceration.

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