Isolation of Soft-Rot Erwinia spp. from Agricultural Soils Using an Enrichment Technique

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ABSTRACT

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A soil enrichment technique was used to isolate soft-rot *Erwinia* spp. from three fallow soils previously cropped to potatoes, a fallowed cabbage soil, soil from a sugarbeet field, and soil from the rhizosphere of volunteer cabbage plants growing among potato plants. Most isolates were classified as *E. carotovora* var. *carotovora* or var. *atroseptica* (sensu Graham). However, all isolates produced blackleg of potato at 18 C and 24 C. The enrichment medium contained: 225 ml distilled water; 0.625 g sodium polypectate; 2.5 ml 10% $(NH_4)_2SO_4$; 2.5 ml 10% K_2HPO_4; and 1.5 ml 5% $MgSO_4 \cdot 7H_2O$. The above mixture was added to 25 g of soil in a 250-ml flask and incubated anaerobically for 48 hours. Cultures were then serially diluted and plated on pectate. Colonies of soft-rot *Erwinia* spp. formed characteristic deep depressions in the pectate surface. The introduced *Erwinia* sp. was recovered 100% of the time from soils artificially infested with greater than 10 cells per gram (dry weight) of soil, 78% of the time from soils containing 2-7 cells per gram (dry weight) of soil, but only 11% of the time from soils containing 0.3 - 1.5 cells per gram (dry weight) of soil.

Additional key words: rhizosphere, soil-borne *Erwinia* spp.

Based on results obtained from the use of selective media, several research workers (2, 6, 8, 9, 11) have concluded that the blackleg organism, *Erwinia carotovora* var. *atroseptica* (L. R. Jones) Holland, and other soft-rot *Erwinia* spp. do not overwinter in field soil. Most recently, Cuppels and Kelman (2) compared a variety of selective media to their crystal violet pectate (CVP) medium and found CVP to be the most efficient for recovery of *Erwinia* spp. from artifically infested soil. However, they were not able to isolate soft-rot *Erwinia* spp. from field soils. In preliminary studies using CVP medium, we also were unable to isolate soft-rot *Erwinia* spp. from either agricultural soils or from soils artifically infested with populations below 1×10^3 *Erwinia* cells per gram (dry weight) of soil. This lower limit of detection corresponds with the results of Cuppels and Kelman (2).

The following report describes an enrichment technique used to isolate low populations of soft-rot *Erwinia* spp. from artifically or naturally infested agricultural soils. A preliminary report has appeared (10).

MATERIALS AND METHODS

Enrichment medium, incubation, and isolation.—To 25 g of soil in a 250-ml Erlenmeyer flask, the following sterilized ingredients were added aseptically in sequence: 225 ml distilled water, 0.625 g sodium polypectate (Sunkist), 2.5 ml 10% $(NH_4)_2SO_4$, 2.5 ml 10% K_2HPO_4, and 1.5 ml 5% $MgSO_4 \cdot 7H_2O$. During the addition of pectate, the soil-water mixture was stirred briskly to minimize coagulation. Remaining visible clumps of pectate were broken-up with a spatula. After addition of salts, the entire mixture was stirred thoroughly to suspend the soil particles. Flasks were incubated anaerobically for 48 hours at room temperature (about 24 C). The pH of the soil mixture ranged from 5.3 to 6.0, depending on the soil used.

Prior to use, soluble sugars were removed from pectate by the following modification of Wood's (16) technique: 500 ml of 60% ethanol (EtOH) were added to 100 g of sodium polypectate and autoclaved for 15-20 minutes. Subsequently, the pectate was washed three times with 250-ml aliquots of 60% EtOH (acidified to 5% HCl), rinsed three times with 150-ml aliquots of 95% EtOH, and dried at 60 C for 12 hours.

After anaerobic incubation, three to five 1-ml aliquots were removed from the flask and serially diluted. Then, three 0.1-ml aliquots of each dilution (1×10^{-2}, 10^{-3}, and 10^{-4}) were plated on pectate (14) and incubated aerobically at room temperature. Before being used, the pectate plates were dried for 2 days at 36 C. Within 2-3 days, pectolytic colonies of *Erwinia* spp. formed characteristic deep depressions in the pectate. Subcultures were transferred to a second set of pectate plates. Colonies which again rapidly hydrolyzed pectate were purified and maintained on nutrient agar (Difco).

Growth of Erwinia species in the enrichment broth.—The growth rate of a potato blackleg-producing *Erwinia* sp. isolate (13) was determined in the enrichment broth under aerobic and anaerobic conditions. Concentrations of nutritents were the same as described above, but sterile tap water was substituted for distilled water.

The *Erwinia* sp. isolate was washed from a 24-hour

yeast-dextrose-carbonate (YDC) agar slant (13) with sterile physiological saline, pelleted by centrifugation, and resuspended twice in saline. The transmittance at 640 nm (Bausch and Lomb Spectronic 20 spectrophotometer) was adjusted to 15.0%. Two milliliters of the suspension were added to 100 ml of the enrichment broth contained in a 500-ml side-arm flask which brought the transmittance to 95%. To achieve anaerobic conditions, cotton-filtered CO_2 was bubbled through the broth for approximately 10 minutes before the flasks were sealed. For aerobic conditions, the side-arm flask was fitted with a sterile cotton plug. All cultures were shaken on a rotary shaker (106 rpm) at room temperature for 48 hours. During this period, the percent transmittance at 640 nm was monitored periodically. Each treatment consisted of two flasks; the experiment was repeated once. Purity of cultures was verified by plating each on nutrient agar at the beginning and end of each experiment.

Enrichment medium sensitivity.—To determine how many *Erwinia* spp. cells could be detected in soil by the enrichment technique, the *Erwinia* sp. isolate, grown on YDC for 2-10 days, was serially diluted in sterile physiological saline and plated on nutrient agar. At the same time, 1-ml aliquots from decreasing dilutions were added to separate 25-g quantities of each of two soil types contained in separate 250-ml Erlenmeyer flasks. All dilutions were replicated twice; the experiments were repeated nine times. Addition of nutrients, anaerobic incubation, and plating procedures were followed as described above.

The soils used were: a sandy loam collected from an agricultural field in Litchfield, Arizona, and a clay loam collected from a rose garden on the University of Arizona campus. Neither soil contained detectable populations of soft-rot *Erwinia* spp. The total population of bacteria in these soils prior to infestation with *Erwinia* sp. was determined by spreading serial dilutions of 1-g samples of each soil onto nutrient agar. Plates were incubated at room temperature and counts were made after 5 days. Organic content of soils was determined using a modification of the Walkey-Black procedure (1).

Isolation and identification of Erwinia from naturally infested field soils.—Soil samples were collected from the University of Arizona Experiment Station at Mesa, and from different commercial agricultural fields in Litchfield, Arizona. Two to five soil samples (each weighing 3-5 kg) were aseptically collected from each field (5 to 25 cm soil depth), placed in new plastic bags, and stored at 13 C. Soil samples were collected on 15 January 1975, and (except for potato soil 2, which was processed approximately 75 days after it was collected) processed over a 3- to 4-week period. Each sample of soil was thoroughly mixed before 25-g subsamples were removed for processing.

Soils from commercial fields had the following cropping history: potato soils 1, 2, and 3–potatoes harvested in the spring of 1974, fallow June, July, and August, planted to lettuce in mid-September, plowed under in mid-December, and planted to potatoes 16-17 January 1975; wheat soil–potatoes harvested in spring of 1974, summer and fall history unknown, planted to wheat in December; cotton soil–cotton in 1974 and fallow in 1975.

Soils collected from the Mesa Experiment Station included samples from a lettuce field, a sugarbeet field, a fallow field containing recognizable cabbage debris, and rhizosphere soil of volunteer cabbage plants in a potato field.

Pectolytic isolates recovered from soils were regarded as being a soft-rot *Erwinia* sp. if they were Gram-negative, oxidase-negative (3), and fermented glucose (0.5% glucose in purple broth base (Difco). Isolates identified as soft-rot *Erwinia* sp. were tested for ability to produce acid from maltose and α-methyl glucoside, reducing substances from sucrose according to Graham (5), and for their pathogenicity to 2- to 3-week-old potato plants (cultivar Norgold Russet). The latter was determined by puncturing stems approximately 1-2 cm from the top with the tip of half of a moist toothpick smeared with cells from 24- to 48-hour nutrient agar cultures. Toothpicks were left in place. For each isolate, two plants were inoculated and placed in moist chambers; one plant was incubated at 16-18 C for 7 days and the other at 24 C for 3 days.

RESULTS

Growth of Erwinia spp. in the enrichment broth.—The aerobic and anaerobic growth rates of *Erwinia* spp. in the enrichment broth are shown in Fig. 1. Stationary growth was reached at approximately 19.5 hours aerobically and at approximately 47.5 hours anaerobically. The maximum growth of the aerated culture (15.5% transmittance at 19.5 hours) corresponded to 2.5×10^{10} cells/ml and 5.4×10^{8} cells/ml (57.5% transmittance at 47.5 hours) for the anaerobic culture. Since the stationary phase of anaerobic growth was reached at 48 hours, subsequent soil cultures were incubated 48 hours.

Sensitivity of the enrichment technique.—The introduced *Erwinia* sp. was always recovered from soils artificially infested with populations greater that 10 cells per gram (dry weight) of soil. Frequency of recovery was approximately 78% from soils containing greater than two but less than seven cells per gram dry weight soil, and

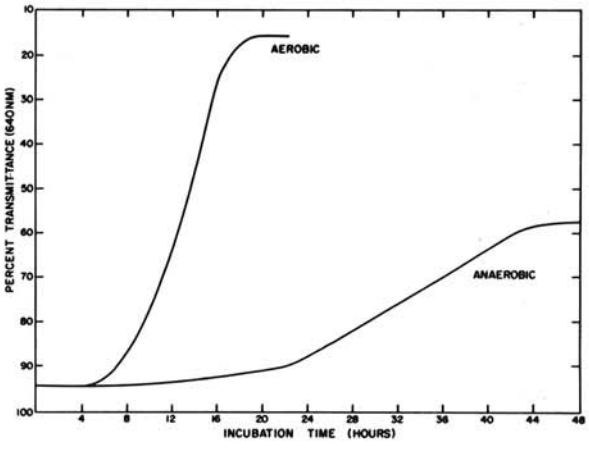

Fig. 1. Effect of aerobic and anaerobic incubation at 24 C on growth of a soft-rot *Erwinia* sp. isolate in pectate enrichment broth.

11% when populations were greater than 0.3 but less that 1.5 cells per gram dry weight soil. When the population of *Erwinia* developed in the enrichment broth, pectolytic colonies were easily recovered at dilutions of 1×10^{-2}, 10^{-3}, and 10^{-4}. No difference was found in the sensitivity of recovery of *Erwinia* spp. from either the field soil (sandy-loam) or rose garden soil (clay loam).

The organic matter, determined on a dry weight basis, was approximately 5.1% in the rose garden soil and 0.22 to 0.34% in the Litchfield soils. The latter value is representative of most agricultural soils in central Arizona (Meneley and Stanghellini, *unpublished*). The total bacterial count in the rose soil ranged from 4.5×10^6 to 9.5×10^6 cells/g (dry weight) of soil, and 7.2×10^6 to 7.7×10^7 cells/g (dry weight) of soil in the Litchfield soil.

Isolation and identification of Erwinia spp. from naturally infested field soils.—Soft-rot *Erwinia* spp. were isolated from six of nine field soils as shown in Table 1. Bacterial isolates that were Gram-negative, fermented glucose within 3 days, hydrolyzed pectate within 2 days, and did not produce cytochrome oxidase, were classified as soft-rot *Erwinia* spp. Of 34 pectolytic *Erwinia* spp. isolated, two possessed the physiological characters of *E. carotovora* var. *atroseptica* [acid from sugars and reducing substances from sucrose (5)], 23 possessed the physiological characters of *E. carotovora* var. *carotovora* [neither acids nor reducing substances produced (5)], and nine could not be identified to variety. Five of the latter isolates, however, produced reducing substances from sucrose. Not all isolates from the same soil sample produced the same biochemical reactions. However, all isolates produced blackening, wilting, and soft-rot of potato stems, which is typical of blackleg, at 18 C and 24 C.

DISCUSSION

With this enrichment technique, sensitivity in isolating soft-rot *Erwinia* spp. from soil has been increased 100- to 1,000-fold over existing isolation procedures (2, 9, 15), thereby facilitating their recovery from naturally infested field soils. Our study showed that recovery of *Erwinia* spp. from a sandy loam with 0.22 to 0.34% organic matter, or from a clay loam with 5.1% organic matter was possible 78% of the time when the population of *Erwinia* spp. was only two-to-seven cells per gram dry weight soil.

The basic principles of enrichment culture is one of natural selection. Organisms that find the medium most suitable for their development will eventually outgrow competitors and predominate in the culture. Presumably, the pectate broth described in this study was not utilized quickly by other soil bacteria, but did provide a carbon and energy source for rapid growth of *Erwinia* spp. Most importantly, the anaerobic conditions eliminated competitive growth of aerobic, pectolytic organisms (primarily fungi and soft-rot *Pseudomonas* spp. while not restricting development of low populations of the facultatively anaerobic, soft-rot *Erwinia* spp. Obviously, the enrichment method does not quantify the population of *Erwinia* spp. in naturally infested soils. An adaptation of the most probable number technique, however, could be used to estimate the soil populations of *Erwinia* spp. Such studies are in progress.

Researchers in Ireland, Scotland, and the northern United States have concluded that soft-rot *Erwinia* spp. do not overwinter in field soils. These judgements may be wrong due to the insensitivity of isolation techniques employed by these researchers. It seems more likely that low temperatures would increase the survival time of

TABLE 1. Isolation of soft-rot *Erwinia* spp. from naturally infested field soils

Source of soil	Soil samples examined[a] (no.)	Soil samples containing soft-rot *Erwinia* spp. (no.)	Varieties (sensu Graham) of *E. carotovora* isolated from soil samples
Potato — 1	3	1	var. *carotovora* var. not identifiable
Potato — 2	3	1	var. *atroseptica*
Potato — 3	5	4	var. *atroseptica* var. *carotovora* var. not identifiable
Wheat	3	0	...
Cotton	3	0	...
Cabbage			
rhizosphere	4	3	var. *carotovora* var. not identifiable
Lettuce	2	0	...
Sugarbeet	2	1	var. *carotovora*
Fallow cabbage	3	3	var. *carotovora* var. not identifiable

[a]Each sample, containing 3-5 kg of soil, was collected from different locations in the field; 25-g subsamples from each collection were used in making determinations.

Erwinia spp. in soil, especially if they were associated with plant debris (9). This is supported by results of Ficke et al. (4) who found that long, frosty periods, snow cover, and straw and plant residues, prolonged the left of *E. carotovora* var. *atroseptica* in soils. Unless soft-rot *Erwinia* spp. are seed-borne or spread from soft-rotted plants, fruits, or vegetables via insects, birds, etc., the ubiquitous occurrence of *Erwinia*-caused soft rots on various crops is not easily explained if it does not survive in soil.

High soil temperatures, greater than 20 C, are presumably inimical to the survival of soft-rot *Erwinia* spp. (11). However, we isolated *Erwinia* spp. from certain soils (potato soils 1, 2, and 3) that had undergone a summer fallow. Admittedly, the surviving populations of soft-rot *Erwinia* spp. might have multiplied on lettuce planted after the summer fallow. However, the initial populations of these bacteria had to survive in soil where summer temperatures ranged from 20 to 31.5 C (average 27.5 C) at the 25-cm soil depth, and 18 to 49.5 C (average 35 C) at the 12.5-cm depth (Meneley and Stanghellini, *unpublished*). Since these infested soils were planted to potatoes within 2 days after the soil samples were collected, the populations of *Erwinia* spp. already present in the soil could serve as inoculum capable of inciting blackleg of potato.

The failure to isolate soft-rot *Erwinia* spp. from lettuce, wheat, and fallowed cotton soils does not necessarily mean the organism is entirely absent from the field. If an *Erwinia* sp. is located in pockets sparsely distributed throughout a field, the chances of detecting those sites are low. Therefore, a number of locations should be sampled before conclusions are made concerning field-wide *Erwinia* sp. infestations. Similarly, pockets of *Erwinia* spp. could serve as inoculum for new infection centers in subsequent crops. Since low populations of soil-borne bacteria can increase rapidly under conducive environmental conditions, spread throughout the field from these foci may occur via water, insects (7), and/or cultural practices and may account for the reported incidence of such diseases as soft-rot of cabbage (15), lettuce (12), sugarbeet (S. V. Thomson, *personal communication*), and the reoccurrence of blackleg of potato despite the use of *Erwinia*-free planting stock (6).

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