A MODEL FOR ECOLOGICAL STUDIES ON SOFT-ROT ERWINIA:
ORIGIN AND SURVIVAL OF ERWINIA CAROTOVORA VAR.
ATROSEPTICA, A PATHOGEN OF MATURE SUGARBEETS

by

Margarida Matos de Mendonça

A Dissertation Submitted to the Faculty of the
DEPARTMENT OF PLANT PATHOLOGY
In Partial Fulfillment of the Requirements
For the Degree of
DOCTOR OF PHILOSOPHY
In the Graduate College
THE UNIVERSITY OF ARIZONA

1978
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THE UNIVERSITY OF ARIZONA, PH.D., 1978
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I hereby recommend that this dissertation prepared under my direction by Margarida Matos de Mendonça entitled A MODEL FOR ECOLOGICAL STUDIES ON SOFT-ROT ERWINIA: ORIGIN AND SURVIVAL OF ERWINIA CAROTOVORA VAR. ATROSEPTICA, A PATHOGEN OF MATURE SUGARBEETS be accepted as fulfilling the dissertation requirement for the degree of Doctor of Philosophy.

Dissertation Director

As members of the Final Examination Committee, we certify that we have read this dissertation and agree that it may be presented for final defense.

Final approval and acceptance of this dissertation is contingent on the candidate's adequate performance and defense thereof at the final oral examination.
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SIGNED: Magarida Felix de Mendonça
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ABSTRACT

Studies on the origin and survival of *Erwinia carotovora* var. *atroseptica* (biotype KSB), a pathogen of mature sugarbeets, were undertaken in the Sulphur Springs Valley (Arizona) after a root-rot was reported in July 1975. The sugarbeet field was plowed in October 1975, fallowed until March 1976, planted to wheat, harvested in August, and subsequently fallowed. Rhizosphere soil from wheat and various weeds was collected monthly from April to August 1976, and assayed, using a soil enrichment technique, for the presence of biotype KSB. Soil and rhizosphere isolates were identified using serological, biochemical, physiological, and sugarbeet pathogenicity tests. Biotype KSB was recovered from the rhizosphere of wheat, various weeds, and volunteer sugarbeets throughout the sampling period (5 months). KSB also was recovered from the rhizosphere of corn plants in another field with the same crop history except that corn was planted in April. Several isolates of *E. carotovora* also were recovered which were serologically reactive with antisera developed for sugarbeet isolates from other geographical regions in the U.S., and with isolates from various other crops which were identified as *E. carotovora* var.
carotovora. Biotype KSB could never be recovered from fallow (weed-free) agricultural soils.

To determine if KSB was indigenous to Arizona in native weeds, rhizosphere soil samples from two regions in the Chiricahua Mountains, a watershed for the Sulphur Springs Valley, were screened. Several Erwinia spp. were isolated from the rhizosphere of Lupinus blumerii Greene. Biotype KSB was among the isolates recovered. Attempts to isolate this organism from other native plants in the same or other (Saguaro National Monument, Tucson) areas were unsuccessful.

Another study was undertaken in a commercial field in Chandler (Arizona), where biotype KSB could not be recovered, to determine the pattern of vertical distribution of the pathogen in the rhizosphere of mature sugarbeets. Biotype KSB was recovered during 4 months from the rhizosphere of artificially infested mature sugarbeets. However, a distinct pattern of vertical distribution was noted with an apparent preference for soil depths greater than 12 cm. The role of temperature and soil matric potential on this distribution was investigated in the laboratory using naturally infested soils containing biotype KSB. Results showed that biotype KSB persisted longest (4 months) when soil temperatures were low (0 and 10 C). High soil moisture also favored survival. The pathogen persisted only briefly
under dry soil conditions or fluctuating temperatures, possibly explaining the lack of recovery of *E. carotovora* var. *atroseptica* from the surface of fallowed soils during summer periods.

An additional factor affecting the survival of biotype KSB was the composition of the substrate utilized for laboratory produced inoculum. Washed cells of biotype KSB, harvested from standard and sucrose supplemented media, during stationary phase, were subjected to starvation in pH 7 phosphate buffer at 25°C. Cells harvested from the sucrose supplemented media attained lower viabilities earlier but persisted longer than those from the standard media.

The ELISA (enzyme-linked immunosorbent assay) technique allowed the detection of 30 KSB cells/ml of a buffer suspension under the limits of visual assessment. With modification this procedure may provide a useful technique for detecting low populations of bacteria.
CHAPTER 1

INTRODUCTION

The study of survival capabilities of plant pathogenic bacteria should provide data useful in determining control measures. Although most phytopathogenic bacteria are nonspore-forming, species apparently endure adverse environmental conditions through the use of one or a combination of specialized survival mechanisms. They include: persistence in intimate association with the host (7, 26), persistence in soil (3, 8) or nonhost materials (15), and the development of specialized relationships with various insects (14). Since a pathogen may persist in several ways, it frequently is difficult to determine which survival mechanism is most effective.

Certain varieties of Erwinia carotovora have been associated with soft-rots of a wide range of field and vegetable crops (23, 28, 35, 36). However, contrary views exist regarding the soil-borne nature of the soft-rot Erwinia (9, 16, 24, 27, 36). Several factors contribute to those differences of opinion. First, numerous techniques have been employed in attempting to detect populations of soft-rot Erwinia in soil (17, 24). Since the sensitivities of the
techniques vary, success in recovery is dependent upon the method used (9, 24). Second, isolates of soft-rot Erwinia recovered from the rhizosphere of cultivated (4, 15, 20) and noncultivated (6, 10) plants have seldom been accurately identified as the same strain or biotype associated with a specific disease of a susceptible cultivated plant. Third, little attention has been paid to the effect of environmental factors (soil moisture, soil temperature, depth of sampling, etc.) and prior nutrition on the survival potential of soft-rot Erwinia.

This investigation was undertaken to study the ecology of *Erwinia carotovora* var. *atroseptica*, which can cause root-rot of mature sugarbeets (33). The occurrence of this disease coincided with the widespread use of virus-resistant sugarbeet cultivars (USH 9 and 10) in California (33), Washington (30), and Arizona (31). Two distinct serological and physiological biotypes of sugarbeet pathogen were found in widely separated production areas. Biotype KSB was associated with the disease in the Sulphur Springs Valley (Arizona), whereas the other biotype, CB, was associated with root-rot in California, Washington, and Chandler (Arizona).

The origin of the sugarbeet pathogen is not known and attempts to demonstrate the seed-borne nature of the bacterium have been unsuccessful (31, 34). Consequently, it has been hypothesized that the pathogen is indigenous to
certain production areas (22, 31, 34). Recovery of the bacterium from soil collected in fields where the disease had previously occurred was often negative (35); therefore, it was suggested that perhaps the sugarbeet pathogen survived in the rhizosphere of nonhost plants (35). However, no direct confirmatory evidence has been presented. On the other hand, the occurrence of other pectolytic *Erwinia* spp. in the rhizosphere of nonhosts is well documented (4, 6, 10, 15, 19, 20, 28), but the significance of this rhizosphere population has not been determined with regard to a specific disease caused by an identifiable member of that population.

The specific objectives of this investigation were to determine: (i) the survival potential of an identifiable biotype of the sugarbeet pathogen (KSB) in the rhizosphere of cultivated and noncultivated plants in the Sulphur Springs Valley; (ii) if vegetation in nonagricultural areas harbors this specific soft-rot bacterium; (iii) the vertical distribution of the pathogen in the rhizosphere of mature sugarbeets; (iv) the influence of soil moisture, soil temperature, and prior nutrition on the survival capability of the bacteria in soil, and (v) the applicability of serological methods in detecting low populations of these bacteria in soil.
CHAPTER 2

ORIGIN AND SURVIVAL OF ERWINIA CAROTOVORA
VAR. ATROSEPTICA FROM SUGARBEETS

Determination of the Survival Potential of E. Carotovora
VAR. ATROSEPTICA in the Rhizosphere of Cultivated and
Noncultivated Plants in Agricultural Areas

Materials and Methods

Isolations were made from soil (rhizosphere and
bulk) collected from two fields (A and B) in the Sulphur
Springs Valley where root-rot of sugarbeets had previously
occurred in 1975. The cropping history of field A was:
sugarbeets harvested in October 1975, fallowed until March
1976, planted to wheat, harvested in August, and subsequently
fallowed. The cropping history of field B was the same
as A except corn was planted in April.

From April to August 1976, 10 to 12 weeds and crop
plants, with adhering rhizosphere soil, were aseptically
collected each month, placed inside new plastic bags, and
transported to the laboratory in ice boxes. Identical num-
bers of nonrhizosphere soil samples were collected at depths
of 0-20 cm, in the vicinity of plants sampled. During fal-
low periods, bulk soil was sampled at depths of 0-20 cm and
in the original sites of rhizosphere sampling.
Rhizosphere and bulk soil isolations were accomplished within 1-3 days after collection as follows: approximately 5 g of roots and adhering soil (for rhizosphere determinations) or bulk soil were placed in 50 ml of sterile distilled water (SDW) and shaken for 5 min. The suspension was then serially diluted with SDW and 0.1 ml portions were spread on the surface of crystal violet pectate (CVP) (9), in triplicate, either directly or after enrichment as outlined by Meneley and Stanghellini (24). After a 48-hr incubation at 25 C, pectolytic colonies were subcultured on m-endo LES agar (Difco) plates, then transferred back to CVP for confirmation. Stock cultures were maintained on nutrient agar (Difco) slants.

Soil and rhizosphere isolates were identified using serological, biochemical, physiological, and sugarbeet pathogenicity tests as described by Stanghellini et al. (31).

Results

The sugarbeet pathogen (biotype KSB) was consistently recovered from the rhizosphere of wheat, various weeds, and volunteer sugarbeets during a 5-month period in field A (Table 1). It was also recovered from the rhizosphere of corn in field B. Additionally, isolates of *E. carotovara* were obtained which were nonpathogenic to sugarbeets, but were serologically reactive with antisera developed to
Table 1. Isolation of soft-rot Erwinia from rhizosphere soil of various weeds and crop plants in agricultural areas (fields A and B)

<table>
<thead>
<tr>
<th>Date of Sampling (1976)</th>
<th>Crop or weed</th>
<th>No. of Plants Sampled</th>
<th>No. of plants harboring Other pectolytic Erwinia spp.</th>
</tr>
</thead>
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<tr>
<td>April</td>
<td>Beta vulgaris L. (volunteer sugarbeets)</td>
<td>7</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>Triticum sp. (wheat)</td>
<td>6</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>Sisymbrium irio L. (London rocket)</td>
<td>7</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td>Miscellaneous weeds</td>
<td>10</td>
<td>-</td>
</tr>
<tr>
<td>May</td>
<td>Triticum sp. (wheat)</td>
<td>5</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>Amaranthus palmeri Wats. (pigweed)</td>
<td>6</td>
<td>3</td>
</tr>
<tr>
<td>June</td>
<td>Triticum sp. (wheat)</td>
<td>4</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>Miscellaneous weeds</td>
<td>8</td>
<td>4</td>
</tr>
<tr>
<td>July</td>
<td>Zea mays L. (corn)</td>
<td>6</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>Triticum sp. (wheat)</td>
<td>4</td>
<td>3</td>
</tr>
<tr>
<td>August</td>
<td>Triticum sp. (wheat)</td>
<td>12</td>
<td>8</td>
</tr>
</tbody>
</table>

a. The crop history of field A was: sugarbeets harvested in October 1975, fallowed until March 1976, planted to wheat, harvested in August, and subsequently fallowed. The crop history of field B was the same as A except corn was planted in April.

b. Identification based on serological, physiological, and pathogenicity tests (31).
isolates from other geographical regions in the U.S., as well as isolates identified as *E. carotovora* var. *carotovora* (31).

The sugarbeet pathogen could not be recovered from fallow soils collected after the wheat harvest in field A.

**Isolation of *E. Cartovora* var. Atroseptica from Native Vegetation in Nonagricultural Areas**

**Materials and Methods**

Native vegetation was collected in July 1977 from two regions: Pinery Canyon (elevation 1,800 m) and Fly's Peak (elevation 2,500 m) within the Chiricahua Wilderness Area of the Chiricahua Mountains, a natural watershed for the Sulphur Springs Valley (elevation 1,200 m). These regions were located 50 km from commercial agricultural enterprises and sugarbeet fields. Native plants (Table 2) and adhering soil were placed in plastic bags and transported to the laboratory in ice boxes. Samples were collected aseptically and processed within 24 hr. Bacterial isolates recovered were identified as previously described.

**Results**

The sugarbeet pathogen (biotype KSB) was consistently recovered from the rhizosphere of *Lupinus blumerii* Greene growing at both elevations (Table 2). Several other unknown serotypes of *E. carotovora* also were recovered.
Table 2. Isolation of soft-rot Erwinia from rhizosphere soil of native vegetation in nonagricultural areas

<table>
<thead>
<tr>
<th>Native plant</th>
<th>No. of Plants Sampled</th>
<th>No. of plants harboring KB(^a)</th>
<th>Other pectolytic Erwinia spp.</th>
</tr>
</thead>
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<tr>
<td>Lupinus blumerii Greene</td>
<td>50(^b)</td>
<td>12(^c)</td>
<td>2</td>
</tr>
<tr>
<td>Oxytropis lambertii Pursh</td>
<td>10</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Calliandra reticulata Gray</td>
<td>5</td>
<td>0</td>
<td>3</td>
</tr>
<tr>
<td>Desmodium batocaulon Gray</td>
<td>10</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Vicia pulchella H.B.K.</td>
<td>6</td>
<td>0</td>
<td>2</td>
</tr>
</tbody>
</table>

a. Identification based on serological, physiological, and pathogenicity tests (31).

b. Two plants collected at Pinery Canyon, 48 at Fly's Peak, Chiricahua Wilderness Area, Chiricahua Mountains.

c. One plant collected at Pinery Canyon, 11 at Fly's Peak, Chiricahua Wilderness Area, Chiricahua Mountains.
Additionally, attempts were made to isolate the KSB biotype from the rhizosphere of native vegetation in non-agricultural areas in the Sulphur Springs Valley and Saguaro National Monument (25 km east of Tucson) in March 1977. Due to the dry soil conditions, rhizosphere samples could be obtained only from the shallow surface layer of soil (0-20 cm). No pectolytic *Erwinia* spp. were recovered from the rhizosphere of native plants in those areas.

**Vertical Distribution of E. Carotovora var. Atroseptica (Biotype KSB) in the Rhizosphere of Mature Sugarbeets under Field Conditions**

**Materials and Methods**

This experiment was conducted in a commercial sugar-beet field of loamy soils which did not contain a natural population of biotype KSB (31), in Chandler (Arizona). Sixty ml of a suspension ($1.4 \times 10^{10}$ cells/ml) of *E. carotovora* var. *atroseptica* (biotype KSB) were dispensed on March 2 in soil adjacent to the crown of 120 healthy, 6-month-old sugarbeets and in four sites of bulk soil located between rows of plants. One row of controls was left between infested sugarbeets. After infestation, four sugarbeets as well as an identical number of control plants were periodically sampled until July, when the entire field was harvested. Sugarbeet plants were carefully removed, placed in individual new plastic bags, transported to the
laboratory in ice boxes, and processed within 24 hr. Rhizosphere soil was removed from the surface of sugarbeet roots at varying distances from the crowns and assayed for the presence of biotype KSB. Populations were estimated by direct plating on CVP, and pectolytic isolates were identified using physiological and serological methods (31). Additionally, bulk soil was collected at 0-15-cm depths, processed as described for infested soil, and assayed for KSB.

Results

The KSB biotype of the sugarbeet pathogen was recovered over a 4-month period from the rhizosphere of mature sugarbeets. However, it was not present throughout the sampling period in the rhizosphere of uninfested plants and infested soil.

Patterns of vertical distribution of KSB populations are presented in Fig. 1.

**Influence of Matric Water Potential and Soil Temperature on Survival of E. Carotovora var. Atroseptica**

Materials and Methods

A naturally infested field soil, collected near the roots of surgarbeets infected with KSB in the Sulphur Springs Valley, was used to determine the effect of various soil matric water potentials and soil temperatures on the
Fig. 1. Vertical distribution of *E. carotovora* var. *atroseptica* (biotype KSB) in the rhizosphere of mature sugarbeets. Erwinia numbers expressed as actual population per sugarbeet sampled.

++ = more than 10 erwinia (KSB)/g soil

+ = less than 10 erwinia (KSB)/g soil

0 = no erwinia (KSB)
Fig. 1. Vertical distribution of *E. carotovora* var. *atroseptica* (biotype KSB) in the rhizosphere of mature sugarbeets. Erwinia numbers expressed as actual population per sugarbeet sampled.
survival of the sugarbeet pathogen. Various samples (20 g) of this soil, at approximately field capacity, were either allowed to air dry and equilibrate with ambient relative humidity (12%) and temperature (25 °C), brought to saturation by the addition of sterile distilled water (SDW), or adjusted to -0.33 bar (Field Capacity, FC) or -15.0 bars (Permanent Wilting Point, PWP) on a pressure plate. The samples were kept at mentioned moisture levels inside petri dishes sealed with masking tape, and incubated at 25 °C.

Total bacterial populations and KSB population were estimated over a 45-day-period. Total bacterial counts were determined according to the method described by Larkin (21) and KSB counts were estimated by plating on CVP (9), in triplicate, with or without enrichment (24), depending on the population.

Twenty-five gram samples of the naturally infested soil mentioned also were placed in sealed petri dishes and incubated at constant temperatures of 0 °C, 10 °C, and 25 °C. Additionally, soil samples were exposed, in the greenhouse, to temperatures fluctuating between 20 °C and 35 °C and to periodic watering to maintain a moisture level near FC. Bacterial populations (KSB) were estimated every 15 days over a 4-month period using the methods described above. All soil studies were repeated once.
Results

Initial KSB populations were estimated to be $6 \times 10^3$, $1.4 \times 10^4$, $2.5 \times 10^4$, and $1 \times 10^3$ colony forming units (CFU)/g of soil that was saturated, at -0.33 bar, at -15.0 bars, and air dried, respectively.

A sharp decline of KSB populations occurred during the first 19 days of incubation, particularly in the last 11 days when populations reached undetectable numbers by direct plating (Fig. 2). After the 19th day, the enrichment technique was used prior to CVP plating. As a consequence, KSB was detected for 36 additional days but only in soils saturated or at FC (Fig. 3). Biotype KSB could not be recovered from soils either air-dried or at -15.0 bars after 19 days. The pattern of survival of the total bacterial population is shown in Fig. 4.

KSB showed a shorter survival period when the infested soil was subjected to fluctuating temperatures, for it was recovered only through 15 days under greenhouse conditions. However, under constant and lower temperatures (25 C), it persisted for 30 days. Throughout a 4-month period, viable cells of KSB declined slowly at 0 and 10 C (Fig. 5).

Discussion

Results of these investigations provide an explanation for the occurrence of root-rot of mature sugarbeets in
Fig. 2. Persistence of *E. carotovora* var. *atroseptica* (biotype KSB) in soils at different matric water potentials. Colony forming units (CFU) obtained by direct plating on crystal violet pectate (9).

---FC = -0.33 bar = Field Capacity; PWP = 15.0 bars = Permanent Wilting Point. Values represent the population in an individual 20-gr sample of soil. This experiment was repeated and gave similar results.
Fig. 3. Persistence of *E. carotovora* var. *atroseptica* (biotype KSB) in soils at different matric water potentials. Colony forming units (CFU) obtained by plating on crystal violet pectate (9) after enrichment (24). --FC = Field Capacity. Values represent the population in an individual 20-gr sample of soil. This experiment was repeated and gave similar results.
Fig. 4. Total viable bacterial counts in soils at different matric water potentials. Colony forming units (CFU) obtained by direct plating on peptonized milk agar supplemented with actidione (21). --FC = Field Capacity; PWP = Permanent Wilting Point.
Fig. 5. Persistence of \textit{E. carotovora} var. atroseptica (biotype KSB) under different soil temperatures. Colony forming units (CFU) obtained by direct plating on crystal violet pectate (9).
certain geographical areas. *Erwinia carotovora var. atroseptica* is indigenous to Arizona on native vegetation, and can persist perhaps indefinitely in agricultural field soils as a rhizosphere inhabitant of both cultivated and noncultivated plants. The existence of a heterogeneous population of soft-rot Erwinia, including the sugarbeet pathogen, in the rhizosphere of cultivated and noncultivated plants in agricultural areas as well as in the rhizosphere of certain native vegetation in nonagricultural areas supports the hypothesis of Stanghellini et al. (31) that there is a "regional selection of a specific biotype of the pathogen by a host from an existing heterogeneous population residing naturally in soil." In addition, the recovery of the pathogen from the rhizosphere of cultivated and noncultivated plants following a 5-month fallow period indicates that this bacterium is capable of persisting in agricultural soils in the absence of any living plant.

The specific ecological habitat of soft-rot Erwinia in soil per se (fallow field soil) is not known. With few exceptions (6, 24), soft-rot Erwinia have not been recovered from fallow field soils (4, 9, 23, 35). Lack of recovery may, in part, be related to the soil depth sampled, in addition to the sensitivity of the detection method employed. Most researchers have attempted to recover soft-rot Erwinia from soil collected at 0-15 cm depth (23, 35, 36). Results
of this investigation, however, indicate that even in the presence of susceptible host, the sugarbeet pathogen exhibits a distinct vertical pattern of distribution in rhizosphere soil, with an apparent preference for soil depths greater than 12 cm. The exact reason for this distribution is not known. Survival capabilities of soil organisms are undoubtedly governed by environmental factors such as soil moisture, temperature, aeration, and nutrient availability. These factors may become limiting to survival, particularly in the surface layer of a fallow field soil. In Arizona, soil temperatures within the surface layer (0-12 cm) in fallowed agricultural fields often exceed 40 C (M. E. Stanghellini, unpublished data). Supposedly, soil temperatures greater than 20 C are inimical to survival of soft-rot Erwinia. Under laboratory conditions, the sugarbeet pathogen persisted longer when soil moisture was high and temperatures were low. The inability of this pathogen to withstand dry environments was also confirmed experimentally. It is not surprising, then, that soft-rot Erwinia have not been recovered from the surface layer of fallowed soils. However, lack of detection does not negate their presence or potential significance. As Buddenhagen (3) states, it might be unrealistic to expect pathogenic bacteria to maintain high survival numbers in soil. Low bacterial populations, once stimulated by root or other plant exudates, can rapidly
increase to sufficient numbers to cause disease on a sus-
ceptible host.

According to Mew (25), a severe outbreak of soft-rot of Chinese cabbage caused by *E. aroidea* (*E. carotovora* var. *carotovora*) occurred in Taiwan, in a field which had not been cropped to a susceptible host for 20 years. The patho-
gen could not be detected in soil before planting, and at-
ttempts to isolate it from cabbage seed were unsuccessful. Similarly, Togashi (36) reported that *E. aroidea*, present in soil at undetectable levels, increased drastically ($10^6$ cells/g soil) only during a specific growth stage of a sus-
ceptible plant. Within one week after removal of the host, the pathogen could not be detected in soil.

Results of this investigation, coupled with previous work, indicate that soft-rot Erwinia are, indeed, soil borne and that the soil population can function as a primary inocu-
lum under suitable environmental conditions. Their ability to multiply rapidly in the presence of a colonizable sub-
strate (36) and effectively compete in a saprophytic manner for root exudates, as evidenced by numerous reports on the occurrence of soft-rot Erwinia in the rhizosphere of a wide variety of plants, indicates that this group of plant patho-
genic bacteria can be classified as true soil inhabitants (13), as suggested by Togashi (36).
CHAPTER 3

EFFECT OF PRIOR NUTRITION ON STARVATION SURVIVAL OF ERWINIA CAROTOVORA VAR. ATROSEPTICA

Introduction

The use of laboratory produced inoculum is a common practice on survival studies of soil borne pathogens (18, 29). Although survival of bacterial populations may depend upon environmental factors as well as the internal makeup of the inoculum, extrapolations from laboratory studies are often made without concern for the effect of prior nutrition on survival.

The present study was undertaken to determine the effect of prior nutrition on starvation survival of E. carotovora var. atroseptica.

Materials and Methods

KSB was grown in a sucrose supplemented (0.5%) substrate (nutrient broth, Difco) and a nonsucrose medium (nutrient broth, Difco). The media were distributed in 100 ml amounts in 250-ml-erlenmeyer flasks. Each flask was seeded with 0.1 ml of a cell suspension of KSB (prepared by removing the growth of a 48-hr-old nutrient agar slant with sterile distilled water) and incubated for 48 hr at 25 C on a New Brunswick gyratory shaker at 220 to 230 cycles per min.
Bacterial cells, in the stationary phase, were then collected by centrifugation at 5,000x g for 20 min at 23 C. The pellets were washed three consecutive times with phosphate buffer, 0.01 M $K_2HPO_4 - KH_2PO_4$ pH7, and finally suspended for the starvation test.

The percentage of viable cells was determined every 24 hr over a 6-day period by a surface plating method. One-tenth ml amounts of appropriate dilutions, in triplicate, were spread over the surface of m-endo LES agar (Difco) plates supplemented with 0.5% sucrose. Colony counts were made 48 hr after incubation at room temperature (25 C). The experiment was repeated once.

**Results**

Viability of KSB cells harvested from the nonsucrose medium decreased to 65% in 2 days, to 4% by day 4, and thereafter approached 0% (Fig. 6). After 5 days, viability of these cells was lost completely. However, KSB cells harvested from the sucrose supplemented medium attained lower viabilities earlier (3% after 48 hr) but persisted for 6 days.

Ability to resist starvation has been related to the presence of cytoplasmic granules of poly-$\beta$-hydroxybutyrate (PHB) (32), a lipid which can be microscopically detected after staining with Sudan Black B (C. E. Kado, Professor of Plant Pathology, Univ. of Calif., Davis, lecture notes). Cells of KSB collected from the nonsucrose and sucrose
Fig. 6. Survival pattern of *E. carotovora* var. *atroseptica* (biotype KSB) in a phosphate buffer after sucrose supplemented and nonsucrose media had been used as substrates. --Counts express averages of triplicate seeding. This experiment was repeated and data from both tests were in agreement. Results of one test are presented.
supplemented media and stained with Sudan Black B revealed the presence of a lipid compound. Attempts were made to assay this compound, following a hypochlorite treatment and an ether-alcohol extraction procedure (32, 38). However, instead of obtaining crotonic acid as a final product (ultraviolet absorption maximum of 235 m\(\mu\)), an unknown compound (peak value at 190 m\(\mu\)) was detected.

The presence of capsular materials has been implicated in the survival of bacteria under conditions of stress (37). KSB cells harvested from the nonsucrose and sucrose supplemented media were examined with the electron microscope, in an attempt to determine whether prior nutrition had any effect on the amount of capsular material accumulated. A well defined capsule was detected on KSB cells (Fig. 7). However, no difference could be observed on cells from the nonsucrose and sucrose supplemented media.

**Discussion**

Survival patterns of KSB populations were influenced by the composition of growth media utilized prior to starvation. Thus, the effect of prior nutrition should be taken into account in quantitative studies designed to determine the survival of soft-rot Erwinia in soil, particularly when laboratory produced populations are used.

A long survival time of KSB under starvation stress was no expected (50% survival time about 56 hr) (11). As
Fig. 7. Capsular material detected on *E. carotovora* var. *atroseptica* (biotype KSB) cells suspended in phosphate buffer. Negative staining prepared with 2% phosphotungstic acid. --33,300x
Ensign (12) reported, some common saprophytes as *Aerobacter aerogenes*, *Azotobacter agilis*, *Pseudomonas aeruginosa*, and *Streptomyces mitis* have been found to have comparable values of 45, 50, 84, and 22 hr, respectively. *E. carotovora* var. *atroseptica* may be, then, moderately resistant to starvation in soil with a behavior similar to other bacterial soil saprophytes. This poses the intriguing question of the physiological basis for resistance to death by starvation of this organism. A critical consideration which requires further investigation is the presence of the unknown compound detected and its role as an endogenous reserve during starvation. In addition, the possibility that the slime layer or capsula observed plays a protective role on survival needs to be experimentally evaluated.
CHAPTER 4

SEROLOGICAL METHODS FOR ECOLOGICAL STUDIES ON ERWINIA CAROTOVORA VAR. ATROSEPTICA

Objective

The purposes of this work were to evaluate two serological methods, enzyme-linked immunosorbent assay (ELISA) (5) and serologically specific electron microscopy (SSEM) (2) for ecological studies on E. carotovora var. atroseptica.

Materials and Methods

The ELISA tests were done in polystyrene plates (Cooke Microliter M29AR) and following a modification to a procedure designed by Clark and Adams (5) (Table 3). KSB population counts were estimated by direct plating on nutrient agar. Results of the ELISA tests were expressed quantitatively by measuring absorbance at 405 nm using a Gilford model 2000 spectrophotometer.

For the SSEM studies, copper grids (300 mesh) were floated on drops containing equal amounts of KSB antiserum (25) (diluted 1:10 and 1:100), a milky suspension of bacteria, and 2% phosphotungstate solution, for 5 min at 25 C. Suspensions of bacteria on phosphate buffer (0.1 m KH$_2$PO$_4$ - K$_2$HPO$_4$, pH = 7) contained: (i) KSB, (ii) mixtures of KSB and CB, (iii) mixtures of KSB and other enterobacteria
Table 3. Procedure used for the ELISA (enzyme-linked immunosorbent assay) tests on E. carotovora var. atroseptica (biotype KSB). Modification of Clark and Adams (5).

Add 200 µl purified γ globulin (anti-KSB) in coating buffer to each well. (Incubate for 6 hr at 37 C.)

wash

Add 200 µl KSB suspensions in SDW. (Incubate overnight at 25 C.)

wash

Add 200 µl phosphatase-labelled γ globulin in PBS-Tween. (Incubate for 6 hr at 37 C.)

wash

Add 300 µl p-nitrophenyl phosphate substrate in diethanolamine buffer. (Incubate for 2 hr at 25 C.)

Stop reaction with 50 µl 3.0 M NaOH

Visual assessment

Photometric measurement at 405 nm
(Escherichia coli, Klebsiella pneumoniae and Salmonella spp.) and (iv) mixtures of soil bacteria, including KSB (population of $10^5$ colony forming units/g soil), obtained from soil extracts prepared according to the one-minute-setting method (1). Control grids were prepared with normal serum and another serum which was not reactive to KSB by microagglutination tests. The grids were scanned on a Hitachi HS-7S electron microscope and representative aspects photographed.

**Results**

ELISA absorbance values for different dilutions of the KSB suspension are given in Fig. 8. Our results showed that it was possible to detect 30 KSB cells/ml of suspension under the limits of visual assessment.

Using SSEM, KSB cells were consistently identified in pure (Fig. 9) or mixed (Fig. 10) preparations because of their peculiar flagellar clumping and capsular reaction. KSB antiserum revealed specificity, even though closely related strains were checked. In soil extracts, however, KSB was not detected. Although numerous soil bacteria were observed, their flagella were notably absent.

**Discussion**

The ELISA technique proved to be sensitive and useful in the detection of low populations of Erwinia.
Fig. 8. ELISA (enzyme-linked immunosorbent assay) absorbance values at 405 nm for different dilutions of *E. carotovora* var. *atroseptica* (biotype KSB). Modification of Clark and Adams' procedure (5).
Fig. 9. Electron micrograph of a pure culture of *E. carotovora* var. atroseptica (biotype KSB) after using serologically specific electron microscopy (SSEM) technique and treatment with antiserum specific to KSB. --36,750x
Fig. 10. Electron micrograph of a mixed culture of *E. carotovora* var. *atroseptica* containing biotypes KSB and CB after using serologically specific electron microscopy (SSEM) technique and treatment with antiserum specific to KSB.

23,500x

A = flagellar clumping of KSB

B = capsular reaction of KSB

C = flagella of CB
Fig. 10. Electron micrograph of a mixed culture of *E. carotovora* var. *atroseptica* containing biotypes KSB and CB after using serologically specific electron microscopy (SSEM) technique and treatment with antiserum specific to KSB.
Although soil extracts were not used, it seems possible that successful results might be obtained in dealing with specific biotypes of *E. carotovora* var. *atroseptica* in naturally infested soil. In addition, this technique may expedite the large scale testing of naturally infested soils. To increase the efficiency of this technique, in those circumstances, future experiments should utilize the concentration procedure described above for the SSEM.

SSEM promised to be an excellent procedure for detection of KSB, when this organism is predominant in bacterial mixtures. However, its possible application to identify bacteria in soil extracts needs further study.
LITERATURE CITED


