Direct Fluorescent Antibody Stain Procedure Applied to Insect Transmission of *Erwinia carotovora*

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Portion of a thesis in partial fulfillment of the requirements of an M.S. degree.

Supported by the College of Agricultural and Life Sciences, University of Wisconsin-Madison and by USDA, CSRS Grant 516-15-124 to Colorado State University.

The assistance of Jacquie Ferris in the laboratory and field studies is gratefully acknowledged. We also appreciate the assistance and advice of K. Kukorowski and K. Chapman, Department of Entomology, University of Wisconsin-Madison and M. Harrison, Department of Botany and Plant Pathology, Colorado State University.

Accepted for publication 19 January 1982.

ABSTRACT

Phillips, J. A., and Kelman, A. 1982. Direct fluorescent antibody stain procedure applied to insect transmission of *Erwinia carotovora*. Phytopathology 72:898-901.

A fluorescent antibody stain (FAS) procedure was used to determine the possible dissemination of *Erwinia carotovora* subsp. *carotovora* (*Ecc*) by insects from a potato cull pile to adjacent potato plants. A serologically distinct strain of *Ecc* that was introduced into the cull pile was identified by the FAS procedure in isolations from insects (usually Diptera), leaves, and daughter tubers. Levels of *Ecc* decreased with increasing distance from the cull pile; however, no symptoms of blackleg or soft rot were observed either on the plants or daughter tubers in the field plot area. The FAS procedure was used to demonstrate insect transmission of *Ecc* from decayed tubers to wounded plants in insect cages. Differentiation of *E. carotovora* subsp. *atroseptica* from *Ecc* was facilitated by use of a solid medium containing tetrazolium chloride and α-methyl glucoside as a carbon source.

The use of fluorescent antibody stain (FAS) techniques for the detection and identification of bacterial pathogens of man and animals is well established (4). The FAS procedure offers many possibilities for the study of the ecology and epidemiology of plant pathogenic bacteria; it has been used to detect soil organisms (3) and certain plant pathogens (1,20,21,22). With these techniques a unique serotype introduced into a specific site can be followed during either its decline or spread into the surrounding area.

The objective of this study was to determine whether the FAS technique can be applied to investigate the role of insects in the dissemination of *Erwinia carotovora* subsp. *carotovora* (*Ecc*). A preliminary report of this research has been presented (19). Two improved procedures are described for selection and identification of strains of *E. carotovora*: a modification of the crystal-violet pectate medium (6), and a tetrazolium chloride plate method for testing utilization of α-methyl glucoside (α-MG).

MATERIALS AND METHODS

Bacteriological techniques. Crystal violet pectate medium (CVP) (6) was modified by adding 0.5 ml of 10% sodium dodecyl (=lauryl) sulfate (SDS) per 500 ml of CVP and by increasing the agar concentration from 0.3% to 0.5% (CVP + SDS). The SDS was added to the CVP before the medium was autoclaved, but after the ingredients were blended. The addition of SDS enhanced the selectivity of CVP in isolations of *E. carotovora* strains from insects.

In the initial evaluation of the CVP medium, 15 g of sodium polypectate was added per 500 ml. Sodium polypectate manufactured by Sunkist Growers, Inc. from a new source (Raltech Scientific Services, 3301 Kinsman Blvd., Madison, WI 53704) was used at a lower concentration (9 g/500 ml) in this modified medium.

The enrichment medium contained 4.5 g of sodium polypectate, 1.0 ml of 0.075% aqueous crystal violet, 0.5 g $NaNO_3$, and 2.25 ml of 1 N NaOH per 500 ml of hot distilled water. The medium was mixed in a blender and then dispensed into test tubes in 1-ml aliquots.

Utilization of α-methyl glucoside. To aid in differentiating *Ecc* from *E. carotovora* subsp. *atroseptica* (*Eca*), a solid medium (α-MG) was developed to test for utilization of α-methyl glucoside. Five different stock solutions were prepared as follows: (A) 0.4% KH_2PO_4, 1.4% K_2HPO_4, and 0.2% NH_4Cl; (B) 0.2% Bacto-casamino acids, 3% Bacto-agar; (C) 10% $MgSO_4 \cdot 7\ H_2O$; (D) 20% α-methyl glucoside; and (E) 1% tetrazolium chloride. These solutions were autoclaved separately and combined prior to pouring the plates. Each batch of α-MG contained 500 ml each of solutions A and B, 1 ml of solution C, 50 ml of solution D, and 2 ml of solution E. Inoculations were made by spot-plating the surface of the α-MG medium. Plates were observed after 3 days at 24 C.

Preparation of fluorescent antibody stain. Ecc strain SR 206 (Dickey 289) isolated from potatoes in Connecticut in 1972 and provided to us by D. Sands was used because it did not react with any of the fluorescent antibody stains (FAS) prepared against a large number of *Ecc* strains from Wisconsin. The criteria established by Dye (8) were used to confirm that this culture was a strain of *Ecc*. To determine whether the antigenically different strain was similar in growth rate to other strains, the generation times of SR 206 and representative Wisconsin *Ecc* strains were determined in shake culture at 18, 24, and 32 C in casamino acids-peptone-glucose broth (CPG) (6) and found to be comparable.

The antiserum was prepared according to the procedures of Allan and Kelman (1) with the following modifications: 1 ml of a glutaraldehyde-fixed bacterial suspension (approximately 10^9 bacteria per milliliter) emulsified in 1 ml of Freund's incomplete adjuvant (Difco Laboratories, Detroit, MI 48201) was injected intramuscularly (IM) into the hind leg of a New Zealand white rabbit. This was followed 3 wk later with a series of four intravenous (IV) injections at 4-day intervals with increasing volumes (0.3, 1.0, 1.5, and 2.0 ml) of the bacterial suspension (14). The rabbit was test-bled 2 days after the last injection, and a large volume of blood was removed 8 days later when the titer was at a maximum level (1,024 at 10^9 cells per milliliter) as determined by

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0031-949X/82/07089804/$03.00/0

standard microagglutination tests. A distilled water suspension of 48-hr *Ecc* cells (SR 206) from CPG medium was used as the antigen for microagglutination tests and the FAS procedure. The FAS, prepared according to the methods of Allan and Kelman (1), had a titer of six.

Cage transmission. Seed corn maggot (*Hylemia platura* Meigen) colonies used in cage studies and maintenance procedures were provided by K. A. Kukorowski of the Department of Entomology, University of Wisconsin-Madison (13).

Stem cuttings from sprouted seed tubers were used as the source of Erwinia-free potato plants. The stem cuttings were prepared as described by Cole and Wright (5). The presence of *Erwinia* in a stem at the time of cutting was determined by squeezing a drop of fluid from the lower end of the stem and spreading the exudate on a plate of CVP + SDS.

The *Ecc* inoculum was prepared by puncturing potato tubers with toothpicks contaminated with *Ecc* strain SR 206, wrapping them in moist paper towels and Saran wrap, and incubating them at 20–24 C for 5 days (7). At the end of this incubation period, tubers usually showed well-advanced symptoms of decay. In the cage-transmission studies, the decayed tubers were cut in half to allow the insects easy access to the decayed tissue.

Six- to 8-wk-old potted potato plants free of *Erwinia* were wounded by cutting off three to four leaves in such a way as to leave a 3- to 4-cm-long area of wounded tissue on the proximal piece of petiole. Five plants were then placed in each of three 46 × 46 × 61 cm insect cages. Twenty-five adult seed corn maggot flies free of *Erwinia* spp. were introduced into each of two cages, one with a rotted tuber in the center and one with no inoculum. No insects were placed in a third cage. Each trial was initiated in late afternoon and discontinued the following day, when the insects were assayed for *Erwinia* as described for the field study. Wounded petiole segments were assayed by following the same procedure used for the insects.

From each plant, two leaves (approximately 4–6 g of fresh tissue) were removed and washed on a rotary shaker for 2 hr in a 125-ml flask containing 50 ml of 0.03 *M* phosphate buffer, pH 7.2, containing 0.1% Bacto peptone (15). After 2 hr, the leaves were removed and 50 ml of the modified pectate enrichment medium (16) was added to each flask. The flasks were incubated without aeration at 20–24 C for 48 hr and assayed for *Ecc* on CVP + SDS.

All isolations of *Ecc* were tested with the FAS for *Ecc* SR 206 to determine whether the *Ecc* strain that was isolated was in fact obtained from the inoculated decayed potato initially placed in the cage.

Field transmission. At the beginning of the growing season, seed potatoes (cultivar Superior) to be used in this field plot were assayed for *Erwinia* by the Saran wrap method (7). Twenty soil samples were taken between 1.5 and 26 m from a cull pile that had been established the previous year. These soil samples were assayed for *Erwinia* with a modified soil enrichment method (16). The cull pile was assayed throughout the season to determine whether *Ecc* SR 206 was present. Decayed tubers were removed; pieces of rotted tissue were suspended in sterile water, and the suspensions were plated on CVP + SDS. All strains of *Erwinia* obtained from these and other assays in this study were tested with the FAS specific for *Ecc* SR 206.

The potatoes were hand-planted in semicircular rows around the cull pile and in straight rows at a distance of 26 m. The planting was located in relation to the cull pile so that prevailing winds would carry aerosols away from rather than to the potato plants (9). The potatoes were planted 0.3 m apart, 17 plants in row 1, 22 in row 2, and 31 in row 3, at distances of 1.5, 3.0, and 4.6 m, respectively, from the cull pile. No insecticide was applied to these three rows of potatoes. At 26 m, four rows, 15 m long, were planted; two of these rows were treated with aldicarb (Temik; Union Carbide, Salinas, CA).

Fourteen kilograms of potatoes that had been inoculated with *Ecc* SR 206 (as in the cage transmission experiments) were then placed on the cull pile to provide a source of inoculum. Ten kilograms of inoculated potatoes were added to the pile in midseason.

At the outset of the study, leaves of the newly sprouted potato plants were swabbed with moist, sterile cotton swabs. Each swab was placed in a separate test tube containing the insect enrichment medium. After 18 hr, each of the swabs was removed, and 0.1 ml of the enrichment solution was plated on CVP + SDS and observed for colonies of *Ecc*. As the season progressed and the plants grew larger, a leaf-wash procedure (15) was substituted for the swabbing technique; in the latter tests, plants were usually assayed in groups of three.

Insects were usually collected in weekly intervals by using a sweep net at various distances from the cull pile. The insects were transferred to plastic bags for transport to the laboratory and placed in a cooler until isolations could be made. The bags were then transferred to a −29 C freezer for 15 min to kill the insects. The normal bacterial flora of the insects was not reduced significantly by 15 min of exposure to this low temperature.

Each insect was identified, transferred to 1 ml of the insect enrichment medium, and crushed with a sterile glass rod. After 12–24 hr of incubation at 20–24 C, 0.1 ml of the enrichment medium was plated on CVP + SDS, incubated and examined for colonies with pits typical of *E. carotovora*. If necessary, these were restreaked on CVP + SDS to obtain pure cultures. Bacteria from isolated *E. carotovora* colonies were spot-inoculated on plates of the α-MG medium.

At the end of the season (8 September), the potatoes were hand-dug and assayed for *E. carotovora* by using the Saran wrap method (7).

Appropriate precautions were taken to insure that the *Ecc* SR 206 from the cull pile was not disseminated inadvertently during sampling. Thus, on a given day, initial samples were taken from the rows at the greatest distance from the cull pile, and final samples were taken near the cull pile. Also, farm and irrigation equipment was not permitted to move through the test area.

RESULTS

Bacteriological techniques. Addition of SDS to CVP enhanced recovery of *Ecc* from insects incubated in the enrichment medium as well as in direct isolations from decayed tubers. The solid tetrazolium chloride medium containing α-methyl glucoside provided an excellent means of differentiating strains of *Eca* and *Ecc*. Twenty-five unknown cultures could be tested per plate. Colonies of *Ecc* were white and slow-growing with a diameter of not more than 3 mm. In sharp contrast, after 3 days *Eca* colonies were deep red with a diameter of 4–6 mm. Thirty stock cultures of *Ecc* and 34 of *Eca* selected at random from our culture collection produced the expected colony appearance on the α-MG medium.

Cage transmission. Insects collected from the cages containing *Ecc* SR 206 inoculum became contaminated with this strain (Table 1). Dissemination to both wounded petioles and leaves occurred, although the percent transmission to the leaves was less than to the wounded petioles (Table 1). No *Erwinia* was detected on plants in the absence of insects.

Field transmission. Assays were negative for SR 206 in samples

TABLE 1. Contamination of insects, petioles and leaves by *Erwinia carotovora* subsp. *carotovora* (*Ecc*) strain SR 206[a] in insect cage studies

Material assayed	Number contaminated per number tested	
	Control	Inoculum present
Insects[b]	0/28	23/63 (37%)
Wounded petioles[c]	0/31	12/31 (39%)
Leaves	0/31	6/31 (19%)

[a] Determined by the fluorescent antiserum staining technique after isolation and biochemical identification of the organism as a strain of *Ecc*.

[b] Twenty-five adult seed corn maggot flies were added to cages containing potato plants with and without inoculum source (control). After one day insects were trapped and tested for *Ecc* SR 206. Not all the insects added to each cage could be trapped. Results represent a summary of three trials.

[c] Leaves were removed so that a 3- to 4-cm-long area was wounded on each petiole. This section of the petiole was removed for sampling.

of soil, insects, and seed potatoes taken before the cull pile was inoculated. After the initial introduction of infected tubers, *Ecc* SR 206 was isolated from the cull pile at each sampling throughout the growing season.

Insects contaminated with *Ecc* SR 206 (average = 7.2%) were collected over the cull pile throughout the season (Table 2). Dipterous insects were the most numerous of the insects collected at the cull pile. A few dipterous insects collected near the cull pile (1.5–4.6 m away) were contaminated, but none collected 26 m from the cull pile were contaminated with SR 206. Other insects collected commonly included potato and aster leafhoppers and potato flea beetles; none of these insects were contaminated with *Ecc* SR 206.

Ambient temperatures at the field plot in May through August averaged 18–24 C; precipitation averaged approximately 6.4 cm in May, June, and August, and 14.0 cm in July. These levels are typical for the potato-growing areas in Wisconsin.

Potato plants planted near (1.5–4.6 m) the cull pile were severely injured by insects; the rows 26 m away treated with aldicarb were relatively free of insect damage; the two untreated rows showed typical insect injury.

Ecc SR 206 was detected on potato leaves from late July until harvest on plants at 1.5–4.6 m, but not at 26 m from the cull pile. Plants in the 1.5-m row had the highest percent of leaves with SR 206 (Table 3). At harvest, *Ecc* SR 206 was detected on some daughter tubers from plants at all four distances (Table 3).

TABLE 2. Number and percentage of insects contaminated with *Erwinia carotovora* subsp. *carotovora* (SR 206)[a] from a field plot in which a potato cull pile was the inoculum source

Insect type		Distance from cull pile (m)		
		0	1.5–4.6	26
Diptera	Number[b]	11/145	2/104	0/37
	Percent	7.6	1.9	0
Potato pests[c]	Number	0/5	0/38	0/322
	Percent	0	0	0
Miscellaneous[d]	Number	2/31	0/83	0/47
	Percent	6.5	0	0
Total	Number	13/181	2/225	0/406
	Percent	7.2 A[e]	0.9 B	0 B

[a] Determined by isolation on CVP + SDS after 24 hr of enrichment and identified with the FAS technique.
[b] Number of contaminated insects per number of insects tested. Collected at weekly intervals (June–August).
[c] Potato leafhopper, aster leafhopper, and potato flea beetle.
[d] Miscellaneous insects included grasshoppers, various Hemiptera, and (occasionally) bees and wasps.
[e] Identical letters in this row indicate no statistical difference ($P = 0.05$).

TABLE 3. Isolation of strain SR 206 of *Erwinia carotovora* subsp. *carotovora*[a] from potato leaves and tubers in relation to proximity to a contaminated potato cull pile[b]

Distance from inoculum source (m)	Number infested		Percent infested	
	Leaves[c]	Tubers[d]	Leaves	Tubers
1.5	7/24	3/86	29.2	3.5
3.0	3/28	5/97	10.7	5.2
4.6	2/36	2/102	5.6	2.0
26.0	0/20	2/167	0	1.2

[a] Determined by enrichment of leaf washings or by the tuber wrapping technique (7), followed by isolation on CVP + SDS and strain identification using FAS.
[b] Potato cull pile containing tubers inoculated with *Erwinia carotovora* subsp. *carotovora* SR 206.
[c] Number of leaf samples contaminated with SR 206 per total number of samples.
[d] Number of tubers infested per total number of tubers sampled.

DISCUSSION

The recovery of *Ecc* strain SR 206 from potato leaves and harvested tubers at levels that decreased with increasing distance from the cull pile indicates that a cull pile may serve as an inoculum source under natural conditions. The use of the FAS technique was effective for obtaining this evidence. Furthermore, the contamination of insects also decreased with increasing distance, indicating that they might be involved with the transmission of the bacterium. This conclusion is supported by the cage studies described above, as well as by the recent work of Kukorowski (13) in Wisconsin and by other investigators (2,10–12,17).

Aerosol dissemination can be another possible mode of spread of *Erwinia* in the field (9,18); and the techniques used to trace spread of SR 206 did not preclude the involvement of aerosol spread. Although the field plot in this study was situated so prevailing winds would carry aerosols away from potato plants, the distances involved were short. Therefore, this precaution may not have been sufficient to preclude spread via aerosols. The use of insect cages in the field was considered as a means of exposing plants to aerosols but not to insects. However, the type of screening needed to exclude small insects was likely to interfere with aerosol deposition on the plants. This facet of the problem would require evaluation before initiation of additional field studies with insect cages in the field.

In cage trials, the seed corn maggot adult transmitted *Ecc* SR 206 to undamaged leaves; hence transmission in the field may have been to undamaged portions of leaves. Once on the leaves, *Ecc* has been shown to persist on foliage under field conditions as detectable, viable populations for several weeks (18).

Caution must be exercised in relating the results of this study with *Ecc* SR 206 to the epidemiology of all *Ecc* strains because of the low numbers of plants and the limited size of the field plots involved. The tubers may have become contaminated in one or more ways: the *Ecc* could have been washed into the soil from leaves contaminated by insects or aerosols, by aerosols or wind-splashed rains from the cull pile to the surrounding soil, or by transmission by egg-laying, root-infesting insects such as the seed corn maggot. However, the fact that *Ecc* SR 206-contaminated tubers were recovered from the field plot planted with seedpieces free of SR 206 supports reports by other workers that *Ecc* can be spread from an inoculum source such as a cull pile to daughter tubers in a potato crop (11,13,18).

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