Overwintering, Seed Disinfestation, and Pathogenicity Studies of the Tobacco Hollow Stalk Pathogen, Erwinia carotovora var. carotovora

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ABSTRACT

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In Connecticut, the tobacco hollow stalk pathogen, *Erwinia carotovora* var. *carotovora,* could not be recovered from soil in the spring in tobacco fields where it had been detected in soil the previous fall. The bacterium was isolated in the spring from decaying tobacco root crowns which had been in the soil since the previous fall. The pathogen could be cultured from tobacco seed stored for 8 mo. A 12-min hot water treatment at 50 C eliminated the pathogen from naturally and artificially infested seeds without affecting seed germination. This bacterium caused hollow stalk symptoms when inoculated into tobacco plants in the greenhouse, but only a few isolates from other *Erwinia* spp. and nontobacco isolates of *E. carotovora* var. *carotovora* caused pith necrosis. Tobacco isolates of *E. carotovora* var. *carotovora* caused necrosis of inoculated tobacco tissue cultures sooner (24 hr) and at lower concentrations (10 bacteria per callus) than did other *Erwinia* strains. Tobacco isolates usually could be separated from nontobacco isolates of *E. carotovora* var. *carotovora* by a positive lipase test on an agar medium.

During the summers of 1974 and 1975, several fields of shade-grown tobacco [*Nicotiana tabacum* L. cultivars Windsor Shade 117 (WS 117) and Consolidated L (Con L)] in the Connecticut River Valley were severely damaged by hollow stalk. This disease, which is caused by *Erwinia carotovora* var. *carotovora* Dye [*Erwinia aroideae* (Townsend) Bergey et al.] is reported to occur on plants in the seedbed (black leg) and in the field (hollow stalk) (14, 20), and on leaves hanging in the curing barn (8, 14). It is usually of minor importance in the field, although occasional serious outbreaks have been reported (14, 21). Experiments were conducted: (i) to determine how the pathogen overwinters and how the disease can be controlled, and (ii) to develop techniques for a study of the pathogenicity of this bacterium to tobacco.

MATERIALS AND METHODS

Isolation and identification.—*Erwinia carotovora* var. *carotovora* was isolated from infected plants or infested soil by placing 10 g of the sample into 100 ml of sterile distilled water, shaking the suspension intermittently for 15 min, spreading appropriately diluted suspension onto the CVP selective medium of Cuppels and Kelman (2), and incubating the plates aerobically or anaerobically (16) (BBl-H_2 CO_2 Gas Pak Anaerobic Systems, Becton, Dickinson, and Co., Cockeysville, MD 21030) in the dark at 30 C. Similarly, tobacco seedlings were assayed by placing 10 seedlings in 10 ml of sterile distilled water, grinding for 5 sec with a glass homogenizer, and spreading appropriate dilutions onto the selective medium. Tobacco seeds were assayed after placing them directly onto the CVP medium. Colonies that were milky white and caused depressions in the CVP medium were streaked onto noninoculated CVP medium and incubated anaerobically as described previously. Single colonies were isolated from these streaked plates. The pathogenicity of these isolates to tobacco was determined and (as described previously) the bacterium was isolated from plants with symptoms. These isolates were subjected to 55 biochemical, physiological, and cultural tests as described in Bergey's Manual (1) to identify *E. carotovora,* and lipolytic activity was determined by the method of Sierra (18).

Pathogenicity studies.—For pathogenicity studies, tobacco isolates of *E. carotovora* var. *carotovora* were adjusted turbidimetrically to 10^7 cells/ml in sterile distilled water. Tobacco plants were grown individually in 240-ml Styrofoam® cups containing a mixture of sterile soil, peat, and sand (1:1:1, v/v). Twelve- to 16-wk-old cultivars WS 117, Con L, and Wisc 38 were inoculated by stabbing the stem with a sterilized needle that had been dipped into the bacterial suspension, or by either removing the top of the plant or a leaf where the petiole joins the stem and placing one drop of inoculum on the wound. Other plants were treated with sterile distilled water or with fresh aqueous suspensions (10^7 cells/ml) of *Erwinia amylovora, Erwinia carotovora* var. *atroseptica,* nontobacco isolates of *E. carotovora* var. *carotovora, Erwinia chrysanthemi,* and *Erwinia stewartii* (Table 1).

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All plants were enclosed in sealed polyethylene bags, placed in the shade in the greenhouse (24 ± 2 C), and observed 7 days later for disease symptoms. The experiment was replicated at least twice with each isolate inoculated on Wisc 38 by placing inoculum onto decapitated plants or onto the site of leaf petiole removal.

The pathogenicity of tobacco isolates of *E. carotovora* var. *carotovora* and selected isolates of other *Erwinia* spp. also was tested on tobacco tissue cultures of Wisc 38. The calli used in these studies had been induced from pith explants 18 mo earlier. Calli (three per plate) were maintained at 25 C in the dark on Linsmaier and Skoog agar medium containing 3% (w/v) sucrose (11). Three to 4 wk after transfer, when calli were 10 to 15 mm in diameter, they were inoculated by placing a 10-μliter drop of water containing 10, 10^2, or 10^3 bacterial cells in the center of each callus. The inoculated calli were incubated in the dark at 30 C and observed 1, 2, 3, and 4 days later for symptoms. The experiment was replicated at least twice with each isolate using three calli per isolate per concentration.

Overwintering.—To determine if *E. carotovora* var. *carotovora* could overwinter in the soil, WS 117 plants with hollow stalk symptoms were cut into 10 to 20 cm long pieces and placed at a depth of 7.5 to 15 cm in the soil of a tobacco field during September 1974. The sites were marked and soil samples were collected in November 1974 and May 1975 and assayed for the presence of the bacterium.

In September 1975, 10 sites were sampled and marked in a heavily cultivated commercial field of WS 117 which had a high incidence of tobacco hollow stalk. Soil samples were assayed for the bacterium at that time and also the following spring (May 1976). In November 1975, 10 root crowns from naturally infected plants were placed in wire baskets and buried 15 cm deep in a tobacco field at the Windsor Station and in April 1976 the decomposing root crowns were collected and assayed.

In September 1974, seeds of WS 117 were collected from plants growing in a field which had a high incidence of tobacco hollow stalk. These seeds were assayed for the bacterium in February 1975. Seeds from this sample also were germinated in a 480-ml plastic tray containing the soil mixture described previously. The container was covered with a polyethylene bag to maintain high humidity and stored at 25 C and with a 12-hr photoperiod supplied by fluorescent lights (260 μEinsteins/m^2/sec PAR). Seedlings free of disease and those with soft-rot symptoms were assayed for the bacterium. Three experiments were performed with seeds from this or other seed lots.

Seeds also were collected in a commercial field from three Con L plants with extensive hollow stalk in September 1975 and assayed for the bacterium. Pieces of plant tissue in the seed lot also were assayed. In January 1976, seed samples (collected September 1975) were obtained from 10 different commercial seed lots and assayed for the bacterium and in June 1976, seed from WS 117 (collected September 1975) was assayed for the bacterium.

Hot water seed treatment.—To determine if hot water treatments would kill *E. carotovora* var. *carotovora* on infested tobacco seeds, it was desirable to obtain seeds with high populations of the bacterium. Seeds of Con L were dipped in a suspension of *E. carotovora* var. *carotovora* (10^7 cells/ml) for 5 min and allowed to air dry on filter paper. About 500 seeds were placed in an Eppendorf pipette tip (VWR Scientific, San Francisco, CA 94119) from which 2 mm of the tip had been removed. The tip of a 10-ml pipette was placed into the large opening of the Eppendorf tip and hot (50 C) sterile distilled water was drawn in to cover the seeds. The small opening was immediately sealed with silicone grease and the Eppendorf tip immersed in a 50-C water bath so that the seeds were below the external water line. Separate samples of seeds were subjected to 50 C for various

TABLE 1. *Erwinia* spp. used in comparative pathogenicity studies on tobacco

Erwinia spp.	Culture collection number	Host	Source
E. carotovora var. *carotovora*	125, 126, 128, 129, 130, 131, 132	tobacco	D. Sands
	105, 106	calla lily	D. Sands
	107	potato	D. Sands
	SR-51	Mexican pepper	A. Kelman
	SR-163	potato	A. Kelman
	EC-14	potato	R. Dickey
E. chrysanthemi	100, 101, 102, 103	poinsettia	D. Sands
	31B	unknown	T. Baker
E. carotovora var. *atroseptica*	111, 112, 113, 114	potato	D. Sands
	SR-8, SR-160, SR-155	potato	A. Kelman
	UR-7	sugar beet	M. Harrison
E. amylovora	135, 136	pear	D. Sands
	27-3	apple	S. Beer
	180-SR	apple	H. Keil
E. stewartii	140, 143, 144 (ATCC 29229, 29230, and 29231, respectively)	corn	D. Sands

intervals from 1 to 30 min, and then immediately placed in cold tap water. Controls were either placed in water for 30 min at 22 C or allowed to remain dry. This experiment was repeated once but the times for hot water treatment varied from 2 to 18 min. In both experiments the cooled, hot-water-treated seeds were air-dried on filter paper and either shaken onto the CVP medium and incubated as previously described, or placed on moistened seed germination paper and incubated under a 12-hr photoperiod (26 μEinsteins/m^2/sec PAR) with 30-C day and 20-C night temperatures. Bacterial growth from seeds was determined after a 2-day incubation and percent seed germination was determined after 5 and 7 days.

Seeds infested naturally (about 1% infestation) with *E. carotovora* var. *carotovora* were placed in 22-C or 50-C water for 12 min. Treated seeds were assayed for the bacterium and their percentage germination was determined as before. Seeds also were sown on the sterile potting mixture and incubated as previously described. These seedlings were observed for soft-rot symptoms and assayed for the bacterium on the selective medium.

RESULTS

Isolation and identification.—*Erwinia carotovora* var. *carotovora* was easily isolated from soil, seeds, and tobacco tissue when the isolation medium was incubated anaerobically. This incubation method excluded growth of pectolytic aerobes, including pseudomonads and bacilli (16).

The strain of *E. carotovora* var. *carotovora* which we isolated from tobacco was very similar to strains from other hosts. Of the 55 tests performed, the most useful differential test was that for lipase; six of eight tobacco isolates precipitated calcium oleate, whereas only one of six strains from other hosts could do so. This exceptional isolate (SR-51 from Mexican pepper) infected tobacco plants, as did the nonlipolytic tobacco isolates.

Pathogenicity studies.—Tobacco isolates of *E. carotovora* var. *carotovora* caused typical hollow stalk symptoms, including pith browning and soft rotting of the stem of WS 117, Con L, and Wisc 38 plants when inoculated on decapitated plants or on the stem at the site of leaf petiole removal (Table 2). Necrosis averaged 11 cm from the injection site 7 days after inoculation. When the pathogen was introduced by stabbing the stem with a needle, it caused pith browning and soft rotting, but not so extensively as that resulting from the other inoculation methods. The treatments with sterile water caused no necrosis. One isolate of *E. chrysanthemi,* the Mexican pepper isolate of *E. carotovora* var. *carotovora,* and two isolates of *E. carotovora* var. *atroseptica* caused symptoms similar to hollow stalk. One isolate of *E. amylovora* caused necrosis of the tobacco tissue but did

TABLE 2. Infection of tobacco cultivar Wisconsin 38 and its calli by isolates of *Erwinia carotovora* var. *carotovora* and other *Erwinia* spp.[a]

		Tissue necrosis at days after inoculation:												
			Callus[c]											
			1 day			2 days			3 days			4 days		
			Bacteria/callus			Bacteria/callus			Bacteria/callus			Bacteria/callus		
Erwinia spp.	Number of isolates	Plant[b]	10	10^2	10^3	10	10^2	10^3	10	10^2	10^3	10	10^2	10^3
E. carotovora var. *carotovora*[d] (tobacco isolates)	8	8	4	4	8	6	8	8	7	8	8	8	8	8
E. carotovora var. *carotovora*[d] (calla lily, Mexican pepper, and potato isolates)	6	1	0	0	2	5	5	5	5	5	5	5	6	6
E. carotovora var. *chrysanthemi*[d]	5	1	0	1	1	2	4	4	4	5	5	4	5	5
E. carotovora var. *atroseptica*[d]	8	0	0	0	1	1	1	1	1	1	2	1	2	3
E. amylovora[e]	4	1	0	0	0	0	0	0	0	0	0	0	0	2
E. stewartii[e]	3	0	0	0	0	0	0	0	0	0	0	0	0	1

[a]Figure represents number of isolates out of those tested causing stem necrosis or callus maceration or necrosis.

[b]Plants (Wisc 38; 12 to 16 wk old) were inoculated with a suspension of bacterial cells (10^7/ml) by decapitating the plant or removing a leaf where the petiole joins the stem and placing one drop of inoculum on the injury site. Symptoms were read 7 days after inoculation.

[c]Tobacco calli, three per plate, were grown on Linsmaier and Skoog's medium and inoculated 3 to 4 wk after transfer by placing a 10 μliter drop of inoculum in the center of each callus.

[d]When disease occurred, these isolates caused browning of the pith and soft rotting of plant-stems and callus maceration.

[e]When disease occurred, these isolates caused moderate stem necrosis of plants and the necrosis and death of the tobacco calli.

not cause typical hollow stalk symptoms.

Tobacco isolates of *E. carotovora* var. *carotovora* caused maceration of tobacco tissue cultures sooner after inoculation and at lower inoculum concentrations than did the other *Erwinia* spp. (Table 2). In fact, 10 and 10^2 cells/callus of four isolates and 10^3 cells/callus of all eight isolates caused callus necrosis 24 hr after inoculation. Several of the soft-rotting *E. carotovora* var. *carotovora* and *E. chrysanthemi* isolates from other hosts caused callus maceration 2 days after inoculation with 10^2 or 10^3 cells/callus, and 83% of the nontobacco isolates of *E. carotovora* var. *carotovora* caused callus maceration 2 days after inoculation with 10 cells/callus. Four days after inoculating calli with 10^3 cells/callus, three of eight isolates of the soft-rotting bacterium *E. carotovora* var. *atroseptica* caused maceration of tobacco calli. Several isolates of the non-soft-rotting bacteria *E. amylovora* and *E. stewartii* caused necrosis but not maceration 4 days after inoculating the callus with 10^3 cells/callus. In all cases, bacterial growth was observed on the callus agar medium only after callus maceration or necrosis had occurred.

Overwintering.—Soil samples collected in November 1974 from areas containing cut-up plant stems all contained more than 100 propagules of *E. carotovora* var. *carotovora* per gram of soil, the lowest detectable level, but the bacterium could not be isolated from any of the soil samples removed from the sample areas in May 1975. In another test, seven soil samples obtained in September 1975 from a commercial tobacco field with a high incidence of hollow stalk contained an average of 500 *E. carotovora* var. *carotovora* per gram of soil and one sample contained more than 30,000 *E. carotovora* var. *carotovora* per gram of soil. This bacterium was not found in the same field in May 1976, although it was recovered in April 1976 from each of the 10 decomposing root crowns.

Erwinia carotovora var. *carotovora* was present on about 1% of the naturally infested seeds which were collected in September 1974 and assayed in January 1975. Tobacco seedlings derived from these seeds exhibited soft-rot symptoms, and *E. carotovora* var. *carotovora* could be isolated only from these diseased seedlings. About 1% of the seeds from the three seed samples collected and assayed in September 1975 were also contaminated with this bacterium, and about 50% of the other plant tissues were contaminated. The same level of contamination was found in two of the 10 seed samples from commercial lots which were assayed in January 1976. Seeds of WS 117 (collected September 1975) also were found in June 1976 to be contaminated (about 1%) with this bacterium.

Hot water seed treatment.—*Erwinia carotovora* var. *carotovora* was not detected on artificially contaminated tobacco seeds after the seeds were treated at 50 C for 12 min (Table 3). Holding the seeds in water at room temperature for 30 min reduced the number of contaminated seeds by 94.3%, and the treatment with water at 50 C for 2, 4, 6, 8, or 10 min caused an average of 99.41 ± 0.2% reduction in the number of contaminated seeds. In our studies, seed germination was not affected by treating seeds up to 18 min at 50 C (Table 3), but 20 min or longer reduced seed germination to 79% or less.

We were unable to detect *E. carotovora* var. *carotovora* on naturally infested seeds which were treated at 50 C for 12 min. The proportion of infested seeds remained at about 1% when treated at 22 C. Some of the seedlings from seeds treated at 22 C, but not at 50 C, exhibited soft-rot symptoms and *E. carotovora* var. *carotovora* could be isolated from these diseased seedlings.

TABLE 3. Effect of hot water treatment (50 C) on numbers of *Erwinia carotovora* var. *carotovora* and seed germination of artificially infested tobacco seeds

Treatment	Period of treatment (min)	Seeds with bacteria[a]		Germination[b]	
		Infested (avg. %)	Clean (avg. %)	Infested (%)	Clean (%)
Dry		100	0	95	97
Room-temperature water	30	5.7	0	95	91
50 C water	2,4,6, or 8	0.6	0	91	92
	10	0.7	0	93	96
	12	0	0	88	87
	14	0	0	92	93
	16	0	0	96	91
	18	0	0	94	89

[a]Seeds of tobacco cultivar Con L were immersed into a suspension of *E. carotovora* var. *carotovora* (10^7 cells/ml), air-dried, and treated in a 50-C water bath. Upon removal from the bath, seeds were cooled in tap water, air-dried, and spread onto the CVP selective medium (2) or onto moistened seed germination paper. An average of 200 seeds were observed for both the contamination and germination studies.

[b]Seeds were germinated in an incubator with a 12-hr photoperiod and 20 C night and 30 C day temperature. Differences were determined after 5 and 7 days. Data presented were obtained on the 7th day.

DISCUSSION

We found no evidence that *E. carotovora* var. *carotovora* overwinters in Connecticut soil. These results are in agreement with those of others who have reported that they were unable to isolate soft-rot *Erwinia* from overwintered field soil (2, 3, 12, 13, 17). However, Meneley and Stanghellini (16) reported that they were able to isolate soft-rot *Erwinia* from field soil in Arizona. Since we could detect up to 30,000 *E. carotovora* var. *carotovora* per gram of soil in September 1975, but were unable to isolate this bacterium from the soil in May 1976, we conclude that the bacterial population was either reduced to levels which could not be detected by our methods, or did not overwinter in Connecticut.

We were able to isolate *E. carotovora* var. *carotovora* from decomposing root crowns which had overwintered in the soil. This suggests that a more complete decomposition or removal of diseased plants prior to fall plowing may reduce the inoculum potential the following spring.

The percentage of contaminated seeds (about 1%) did not decrease from the time of harvest until January the following year, and we were able to recover the bacterium

from seed after they had been stored for 8 mo. This is the first report that the hollow stalk pathogen is seedborne. We also observed that a high percentage of plant debris present in seed samples was contaminated with this bacterium.

Some seedlings derived from infested seed developed soft-rot symptoms. The disease apparently started on a few seedlings and within several days spread to the majority of the seedlings. *Erwinia carotovora* var. *carotovora* could be isolated from these seedlings, but not from seedlings without symptoms. This indicates that infested seed may serve as an inoculum source in the seed bed. We may have been unable to detect this bacterium on symptomless plants because the population was too low, or it was not present on those seedlings. When tobacco seeds were germinated under less humid conditions than those described for these studies, seedlings did not develop soft-rot symptoms.

Epiphytic populations of *E. carotovora* var. *carotovora* have been demonstrated on tobacco leaf surfaces (19) and *E. carotovora* has been isolated from tissues of symptomless plants (9, 15). Since tobacco seedlings derived from infested seeds could develop soft-rot symptoms and *E. carotovora* var. *carotovora* could be isolated from these seedlings, we suggest that symptomless plants may harbor low epiphytic populations of this bacterium until environmental conditions are optimal for disease development. This could explain why symptoms often originate midway up the stem of a mature plant (14). If the organism was entirely soil-borne, one might expect symptoms to develop from lower portions of the stem.

Hot water treatment (50 C, 12 min) killed *E. carotovora* var. *carotovora* on naturally and artificially infested seeds, but did not reduce the germination of treated seeds. In fact, germination was not affected when seed was treated for 18 min at 50 C, which provides a safe margin for the use of this technique as a practical means of disinfesting tobacco seeds. Hankin and Sands (5) also have demonstrated the elimination of this bacterium from tobacco seeds by microwave treatment.

The population of *E. carotovora* var. *carotovora* on artificially infested seeds was reduced greatly by placing the seeds at 22 C, but this reduction did not occur on naturally infested seeds. This indicates that on naturally infested seeds the bacterium is probably associated with grooves and nicks on the seed coat, or is internal to the seed coat.

In greenhouse tests all tobacco isolates of *E. carotovora* var. *carotovora* caused typical hollow stalk symptoms, but few isolates from other plants or isolates of other *Erwinia* spp. caused necrosis of tobacco tissue. All isolates of *E. carotovora* var. *carotovora* and *E. chrysanthemi* caused necrosis of tobacco calli. However, *E. carotovora* var. *carotovora* isolates from tobacco caused callus necrosis sooner and at lower concentrations than did the other isolates. *Erwinia carotovora* var. *atroseptica, E. amylovora,* and *E. stewartii* caused little damage to tobacco calli, and when damage did occur, it was after exposure for several days and at high inoculum concentrations.

We used tissue culture reactions to determine pathogenicity of *E. carotovora* var. *carotovora* to tobacco. Others have not been able to do so with pseudomonads (4, 10). Perhaps the observed differences may reflect the fact that the pseudomonads studied were vascular and leaf pathogens which attack tissues very different from the pith-derived callus tissue. The hollow stalk pathogen normally invades pith tissue and callus invasion might be expected. In addition, Helgeson et al. (6, 7) were able to differentiate tobacco calli derived from plants which were resistant or susceptible to the black shank fungus, *Phytophthora parasitica* var. *nicotianae,* which also invades the pith of susceptible tobacco cultivars. Tissue culture methods may provide a means to select clones resistant to tobacco isolates of *E. carotovora* var. *carotovora.*

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