Galls of *Gypsophila paniculata* Caused by *Erwinia herbicola*

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ABSTRACT

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Galls resembling crown gall disease were observed on *Gypsophila paniculata* nursery cuttings and field-grown plants. No virulent *Agrobacterium* strains were recovered from the galled tissues, and tests for the presence of opines in the galled tissues were negative. Two species of *Erwinia* (*E. herbicola* and *E. rhapontici*) were consistently isolated in high numbers from galls, and *E. herbicola* was isolated in high numbers from leaves and stems. Strains of *E. herbicola* from galls and aerial plant parts of *Gypsophila* induced galling on inoculated cuttings. However, the type culture of *A. gypsophilae* (*E. herbicola* ATCC 13329) was nonpathogenic in our tests. The possible involvement of *E. rhapontici* in the browning and rotting of the *E. herbicola*-induced galls is discussed.

Crown and root galls on *Gypsophila* were first described in the 1930s by Brown (3,4). The causal organism was described as a yellow, facultatively anaerobic bacterium that was first named *Bacterium gypsophilae* Brown (4), later changed to *Agrobacterium gypsophilae* (Brown) Starr & Weiss (25), and now classified as *Erwinia herbicola* (Lohnis) Dye (13,15). However, there are conflicting reports on the ability of these isolates to induce galls on *Gypsophila* (8,12,13,16,26). Crown gall caused by *A. tumefaciens* (Smith & Townsend) Conn has also been reported on this host (26), although attempts to induce galls with the wide-host-range strain B6 of *A. tumefaciens* were not successful (7).

A serious gall disease of *Gypsophila paniculata* L. was brought to my attention by a southern California grower in 1984. Galls were present on rooted cuttings purchased from California and Florida nurseries and on mature field-grown plants in San Diego County. This paper provides evidence that these galls were caused by *E. herbicola* and not *A. tumefaciens*.

MATERIALS AND METHODS

Bacterial isolations. Galled tissues, stems, or leaves from rooted nursery cuttings or field-grown *Gypsophila* plants were washed briefly in tap water and comminuted in sterile distilled water. The suspensions were streaked or plated in dilutions onto the MS (Miller-Schroth) selective medium (17) for isolation of *Erwinia* and onto the *Agrobacterium*-selective media 1A, 2E, and 3DG (14), the medium of Schroth et al (22), or the medium of New and Kerr (18).

The following tests for identification of *E. herbicola* and *E. rhapontici* isolates were performed as described by Schaad (21): Gram stain, silver nitrate flagellar stain, catalase, oxidase, observation of pigments on yeast extract-dextrose-calcium carbonate (YDC) agar, pectate degradation on Hildebrand's medium A, growth at 36 C on YDC agar, potato rot, and gelatin liquefaction. Tests for anaerobic growth, gas production from glucose, and acid production from carbon sources were done in Dye's medium C (9). Tests for H_2S from cysteine, reducing substances from sucrose, and nitrate reduction were also as described by Dye (9). Phenylalanine deaminase and indole tests were performed as described by Smibert and Krieg (23) with the addition of 0.5% yeast extract in the indole test medium. Growth in medium containing potassium cyanide was tested as described by Edwards and Fife (10), but the peptone was reduced from 0.3 to 0.1%; this concentration gave a more definitive reaction between positive and negative bacterial strains.

All strains of *E. herbicola* and *E. rhapontici* described were isolated in this study except *E. herbicola* ATCC 13329, which was obtained from the American Type Culture Collection. *A. tumefaciens* B6 and C58 were obtained from L. W. Moore, Oregon State University.

Plant inoculations. Inoculations were performed on cuttings taken from plants free of *E. herbicola* derived from meristem tip culture (Van Gundy Plant Labs, Riverside, CA). The stock plants for cuttings were maintained in a greenhouse separate from that where inoculations were made. Cuttings were dipped in suspensions of bacterial inocula (10^8–10^9 cells per milliliter) and planted in 2-in. pots with a 1:2 ratio of perlite and U.C. soil mix (1). The cuttings were placed on a greenhouse bench for the duration of the study with intermittent misting for 30 sec every 10 min for 12 hr/day.

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Paper electrophoresis for detection of opines. Plant tissues (150 mg/sample) were extracted with 300 μl of absolute ethanol as described by Kerr and Brisbane (14). The extracts were spotted (2–10 μl) onto Whatman 3MM paper (20 × 46 cm) and allowed to dry. Methylene green was spotted as a visual marker (19). The paper was wetted in the formic acid/acetic acid running buffer (pH 1.8) of Otten and Schilperoort (19) and submerged in carbon tetrachloride in a Pyrex baking dish. The ends of the paper were submerged in running buffer in 500-ml buffer chambers with submerged electrodes. A cooling coil made from Tygon tubing was placed over the submerged paper in the carbon tetrachloride, and cool tap water was circulated through the coil throughout electrophoresis. Electrophoresis was at 25V/cm for 110 min. The paper was removed from the apparatus, and the carbon tetrachloride was allowed to evaporate. The paper was then oven-dried at 55–60 C for 20 min and stained with phenanthrenequinone for mono-substituted guanidines, such as arginine, octopine, and nopaline (19,27). After photographing with shortwave ultraviolet light with Polaroid Type 55 film and Wratten nos. 9 and 23A filters, the paper was stained with silver nitrate for agropine and other silver nitrate-positive opines (5,6). Paper stained with silver nitrate was photographed under tungsten flood lamps with Polaroid Type 55 film.

RESULTS

Description of disease symptoms. Soft, light brown galls (1–2 cm in diameter) were observed at the pruned ends of rooted cuttings of *G. paniculata* obtained from California and Florida nurseries in 1984 and 1985 (Fig. 1A). Galls were present on more than 90% of the cuttings in some lots produced during the spring and summer but on fewer than 20% of those produced during the winter. Field-grown plants developed large galls up to 10 cm in diameter at or below the soil line (Fig. 1B). These galls developed mostly during the spring and early summer and were soft and friable. The galls gradually rotted away during the late summer and winter, often accompanied by stem rot. Galled plants were usually stunted, and plants with gall and stem rots often wilted and died. Losses of 30–60% of *Gypsophila* plants were observed in some commercial fields by the second growing season.

Bacterial isolations. Although many bacteria were recovered from galled tissues of field-grown plants on the selective media for *Agrobacterium*, most of these were later identified as *Erwinia* or *Pseudomonas*. Four colonies resembling *Agrobacterium* were obtained from the medium of Schroth et al or from medium 1A, both selective for biovar 1 of *Agrobacterium*. These isolates were positive for the 3-ketoglycoside test (2), confirming their identification as biovar 1 of *Agrobacterium*. On MS medium, selective and differential for bacteria in the Enterobacteriaceae, colonies resembling *Erwinia* were recovered in high numbers from galls on cuttings, from galls on field-grown plants, and from leaves and stems of field-grown plants (Table 1). Two colony types were consistently recovered on MS medium from galled tissues: large, orange, mucoid colonies and smaller, orange, more restricted colonies. Strains purified from the large colony type were identified as *E. rhapontici*, and strains purified from the smaller colony type were identified as *E. herbicola* (Table 2). Only the smaller (*E. herbicola*) colonies were recovered on MS medium from leaves and stems.

Plant inoculations. None of the four *Agrobacterium* strains isolated from *Gypsophila* galls reproduced gall symptoms when used to inoculate the wounded ends of *G. paniculata* cuttings or wounded tomato or sunflower seedlings. Similarly, strains B6 and C58 of *A. tumefaciens* did not induce galling on *Gypsophila* cuttings. Three of four strains of *E. herbicola* isolated from galls and aerial plant parts of *Gypsophila* induced light brown galls within 2–3 wk on *G. paniculata* cuttings that were characteristic of the natural infections observed on cuttings at commercial nurseries (Fig. 2A). One such gall that was allowed to develop on an inoculated plant for several months attained a diameter of 4–5 cm. *E. herbicola* was reisolated from galls induced by inoculations, and these isolates again produced gall symptoms when used to

Table 1. Populations of *Erwinia herbicola* and *E. rhapontici* in colony-forming units (cfu) per gram of *Gypsophila* tissue

Plant material	Populations recovered on MS medium[a] (cfu/g)	
	E. herbicola	*E. rhapontici*
Galls on nursery cuttings	4×10^7	2×10^7
Galls on field-grown plants	6×10^6	4×10^6
Leaves and stems of galled field-grown plants	3×10^6	0

[a] Selective medium of Miller and Schroth (17).

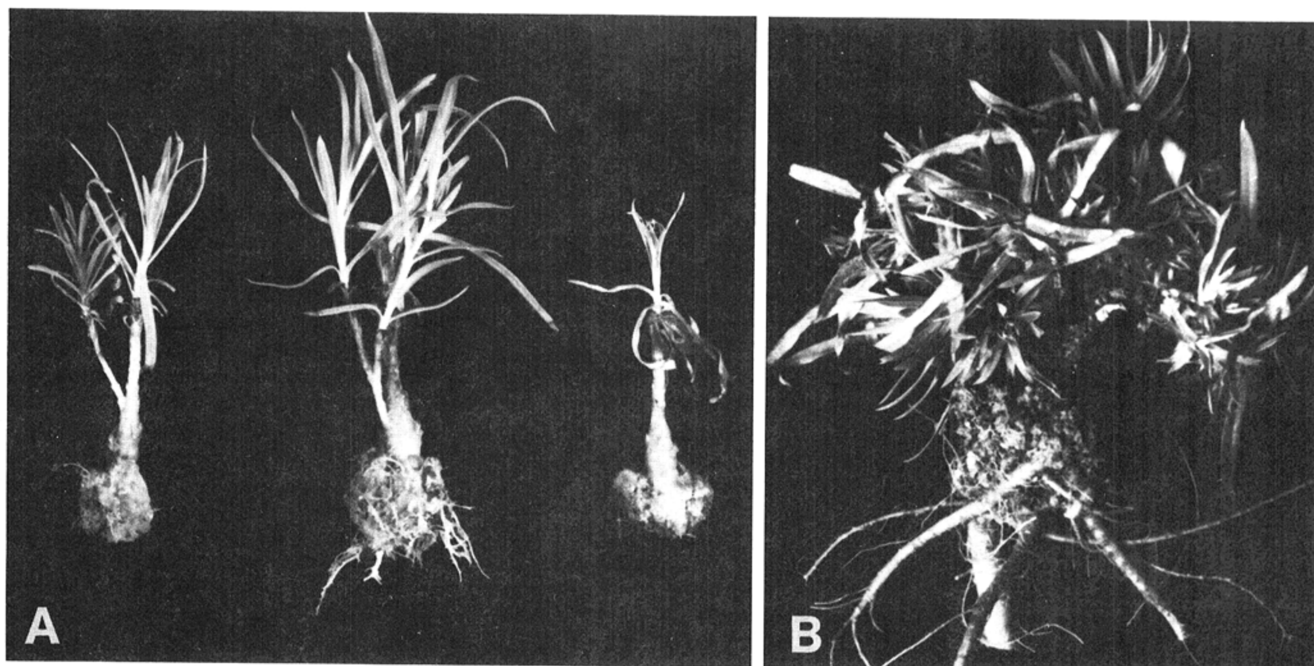

Fig. 1. Gall symptoms on commercial *Gypsophila paniculata*: **(A)** nursery cuttings and **(B)** mature field-grown plant.

reinoculate cuttings. No galls were observed on noninoculated cuttings (Fig. 2B), and *E. herbicola* was not recovered when isolations were performed from these cuttings. Inoculation of wounded aerial stems and leaves with *E. herbicola* also induced swellings (Fig. 2C). ATCC 13329 of *E. herbicola* did not induce galling when used to inoculate *Gypsophila* cuttings.

None of the *E. rhapontici* isolates induced galling. However, *E. rhapontici* was consistently recovered from the brown galled tissues produced by *E. herbicola* inoculations on cuttings maintained on the mist bed in our greenhouse. No *E. rhapontici* was recovered from the propagation stock derived from tissue culture before placing cuttings onto the mist bed, indicating that the *E. rhapontici* was probably surviving on the mist bed as a contaminant from earlier inoculations and rapidly colonizing new plants that were placed on the mist bed. The mist bed was disinfested with sodium hypochlorite to attempt to eliminate *E. rhapontici* contaminants, and inoculations with *E. herbicola* then produced white galls after 2 wk.

Table 2. Identification of *Erwinia* isolates from *Gypsophila* as *E. herbicola* and *E. rhapontici*

	Number of strains with positive reactions[a]				
Test	*E. herbicola* from *Gypsophila* (4 tested)	*E. herbicola* ATCC 13329 (1 tested)	*E. herbicola*[b] (1 tested)	*E. rhapontici* from *Gypsophila* (4 tested)	*E. rhapontici*[b] (1 tested)
Orange colonies on MS[c]	4	1	1	4	1
Gram stain	0	0	0	0	0
Anaerobic growth	4	1	1	4	1
Peritrichous flagella	4	1	1	4	1
Yellow pigment	4	1	1	0	0
Pink pigment	0	0	0	1	1
Oxidase	0	0	0	0	0
Catalase	4	1	1	4	1
Mucoid growth on 5% sucrose	4	0	V	3	1
Growth at 36 C	4	1	1	4	V
H_2S from cysteine	4	1	1	4	1
Reducing substances from sucrose	1	1	V	3	V
Urease	0	0	0	0	0
Pectate degradation	0	0	0	0	0
Potato rot	0	0	NR	0	NR
Gas from D-glucose	0	0	0	4	0
Growth in KCN broth	0	0	0	4	1
Gelatin liquefaction	4	1	1	0	0
Phenylalanine deaminase	4	1	1	0	0
Indole	0	0	0	0	0
Nitrate reduction	4	1	1	4	1
Acid from					
Starch	4	1	1	4	1
Glucose	4	1	1	4	1
Sucrose	4	1	1	3	1
Fructose	4	1	1	4	1
Galactose	4	1	1	4	1
Maltose	4	1	1	4	1
Lactose	4	0	V	4	1
Cellobiose	4	0	0	4	1

[a]V = variable (11–89% of strains are positive) and NR = not reported.
[b]Results for *E. herbicola* and *E. rhapontici* as reported by Krieg and Holt (15).
[c]Selective medium of Miller and Schroth (17).

Paper electrophoresis for detection of opines. Further evidence that the *Gypsophila* galls were not caused by *Agrobacterium* was obtained by analyzing tumor extracts for the presence of opines characteristic of crown gall tissues. After high-voltage paper electrophoresis and staining with phenanthrenequinone, a guanidine compound was detected from both galled and healthy *Gypsophila* tissues that migrated similarly to the arginine control, but no spots were detected in the area where octopine or nopaline were expected to migrate except for weakly fluorescent spots that were present in both gall and healthy root extracts (Fig. 3A). The silver nitrate stain also failed to detect agropine or other silver nitrate-positive opines in *Gypsophila* gall tissues (Fig. 3B).

DISCUSSION

E. herbicola strains isolated from *Gypsophila* galls and aerial stems and leaves induced gall formation on *G. paniculata* when used to inoculate cuttings or wounded stems and leaves. No virulent *Agrobacterium* strains were recovered from *Gypsophila* galls, and no opines were detected in the galled tissues. Therefore, the galls observed on *Gypsophila* nursery cuttings and commercial field plantings were probably caused by *E. herbicola* and not *A. tumefaciens*. There is one abstract reporting that crown gall caused by *A. tumefaciens* occurs on *Gypsophila* (26). However, in the extensive host range study of De Cleene and De Ley (7), the wide-host-range strain B6 of *A. tumefaciens* did not induce tumors on

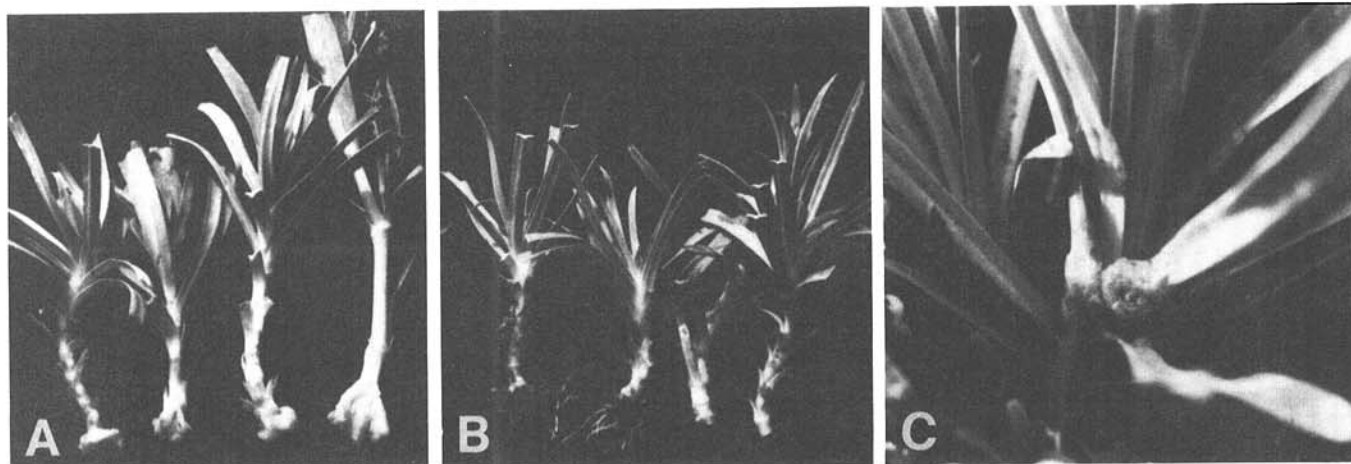

Fig. 2. Galls induced on *Gypsophila paniculata* by inoculation with *Erwinia herbicola*. **(A)** Cuttings inoculated with *E. herbicola* at pruned ends. **(B)** Noninoculated cuttings (same magnification as A). **(C)** Aerial gall produced by inoculating *Gypsophila* leaf with *E. herbicola*.

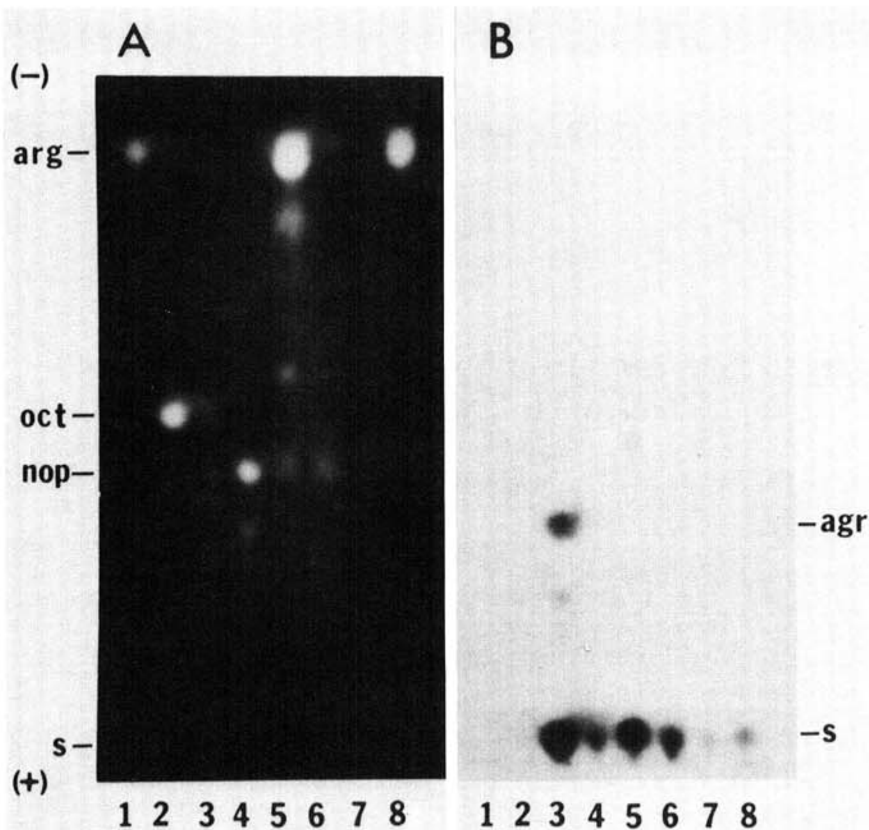

Fig. 3. Paper electrophoresis of gall extracts for detection of opines. **(A)** Phenanthrenequinone stain showing (1) 2 μg of arginine (arg) control, (2) 1.5 μg of octopine (oct) control, (3) 10 μl of extract from an octopine/agropine (agr) tumor induced by *A. tumefaciens* B6 on tomato, (4) 3 μl of extract from a nopaline tumor induced by *A. tumefaciens* C58 on tomato, (5) 5 μl of extract from healthy *Gypsophila* shoot material, (6) 5 μl of extract from a natural gall on a *Gypsophila* nursery cutting, (7) 5 μl of extract from a natural gall on field-grown *Gypsophila*, and (8) 5 μl of extract from a healthy root of greenhouse-grown *Gypsophila*. **(B)** Silver nitrate stain of the same paper showing the silver nitrate-positive agropine from the control *A. tumefaciens* B6 tumor extract but no silver nitrate-positive opines in *Gypsophila* gall extracts. Migration of opines during electrophoresis was from the start (s) toward the cathode (−).

Gypsophila, and in our studies, strains C58 and B6 did not cause galls on this host. It seems likely that strains of *A. tumefaciens* could evolve that would cause crown gall on *Gypsophila*, but in our search of the literature, we found little evidence for *A. tumefaciens*-induced tumors on *Gypsophila*. The conflicting reports on the pathogenicity of *E. herbicola* on *Gypsophila* (8,12,13,16,26) may have resulted from the loss of pathogenicity in older cultures such as the type culture of *A. gypsophilae* (*E. herbicola* ATCC 13329) which was nonpathogenic in our tests.

The isolation of pathogenic *E. herbicola* in high numbers from aerial leaves and stems helps explain why cuttings taken for propagation develop the disease so readily. The apparent lack of infection on cuttings taken during the winter probably reflects environmental conditions unfavorable to the epiphytic colonization of *Gypsophila* leaves and stems by *E. herbicola*. Control of the disease has been reported using calcium hypochlorite or other chemical treatments to reduce populations of the pathogen on the surfaces of cuttings (20). Such measures are being investigated for control of the disease in California. The use of pathogen-free propagation stock generated through tissue culture is also promising; however, pathogen-free stock obtained from either chemical treatment or through tissue culture could become galled when planted in infested fields. The epiphytic survival of *E. herbicola* on many plants (24) may make eradication of pathogenic strains in the field very difficult.

The relationship between *E. herbicola* and *E. rhapontici* and the development of browning and rotting symptoms of *Gypsophila* galls is worthy of further investigation, because both species were consistently isolated from galls on nursery cuttings and field-grown plants. Brown (4) reported that galls produced by inoculation did not develop the characteristic browning found in natural infections. Although our initial inoculations with *E. herbicola* always produced brown galls, we also consistently recovered *E. rhapontici* from these tissues. After disinfesting our mist bed where inoculations were performed, we were able to induce white galls by inoculation with *E. herbicola*. Further investigations are needed to determine the role of *E. rhapontici* and other soil microorganisms, such as *Phytophthora* (11), in the browning and rotting of *Gypsophila* galls and stems in nursery cuttings and field-grown plants in California.

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