Leaf, Stem, Crown, and Root Galls Induced in Chrysanthemum by Agrobacterium tumefaciens

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ABSTRACT

Leaf, stem, crown, and root galls induced by a chrysanthemum strain of *Agrobacterium tumefaciens* occur sporadically on chrysanthemum (*Chrysanthemum morifolium*). Bacterial isolates from leaf, stem, crown, and basal stem galls were morphologically and physiologically similar in comparative bacteriological tests for this organism, and gave identical pathogenic response when inoculated to many chrysanthemum cultivars of varying susceptibility. *A. tumefaciens,* strain B6, while morphologically similiar to the chrysanthemum isolates, was less pathogenic to chrysanthemum, infecting approximately 50% of the cultivars which were infected by the chrysanthemum strain of the bacterium.

Other isolates of the crown gall bacterium did not produce galls on chrysanthemum.

A. tumefaciens is systemic in chrysanthemum. The bacteria moved upward and downward through the stems from the original inoculation sites, and were repeatedly recovered from inside stems, leaf petioles, and leaf midveins. Primary galls occurred at terminal pinches, the site of lateral shoot removal, or at sterile needle punctures on the stems, leaf petioles and leaf veins. Galls occurred only at the site of an injury if bacteria were present.

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Additional key words: pathogenicity, systemic reaction.

Tumors or galls on the basal stems of chrysanthemum were noted by Smith and Townsend in 1907 (17). They referred to the organism causing the galls as *Pseudomonas tumefaciens*. Since that time, crown gall has been listed in most texts and references as a disease of chrysanthemum; however, the disease apparently has been of minor economic significance, and no specific reports occur in the literature describing in detail the symptoms of crown gall on chrysanthemum. In 1964, Cumming in "The Chrysanthemum Book" referred to a bacterial crown gall caused by *Erwinia tumefaciens* which produced tumors at the base of plants and on roots and caused spindly shoots on stunted plants (5). In 1966, Jones (9) reported that crown gall on chrysanthemum, caused by a bacterium, is confined to the soil level and the symptoms are the formation of a cluster of shoots not

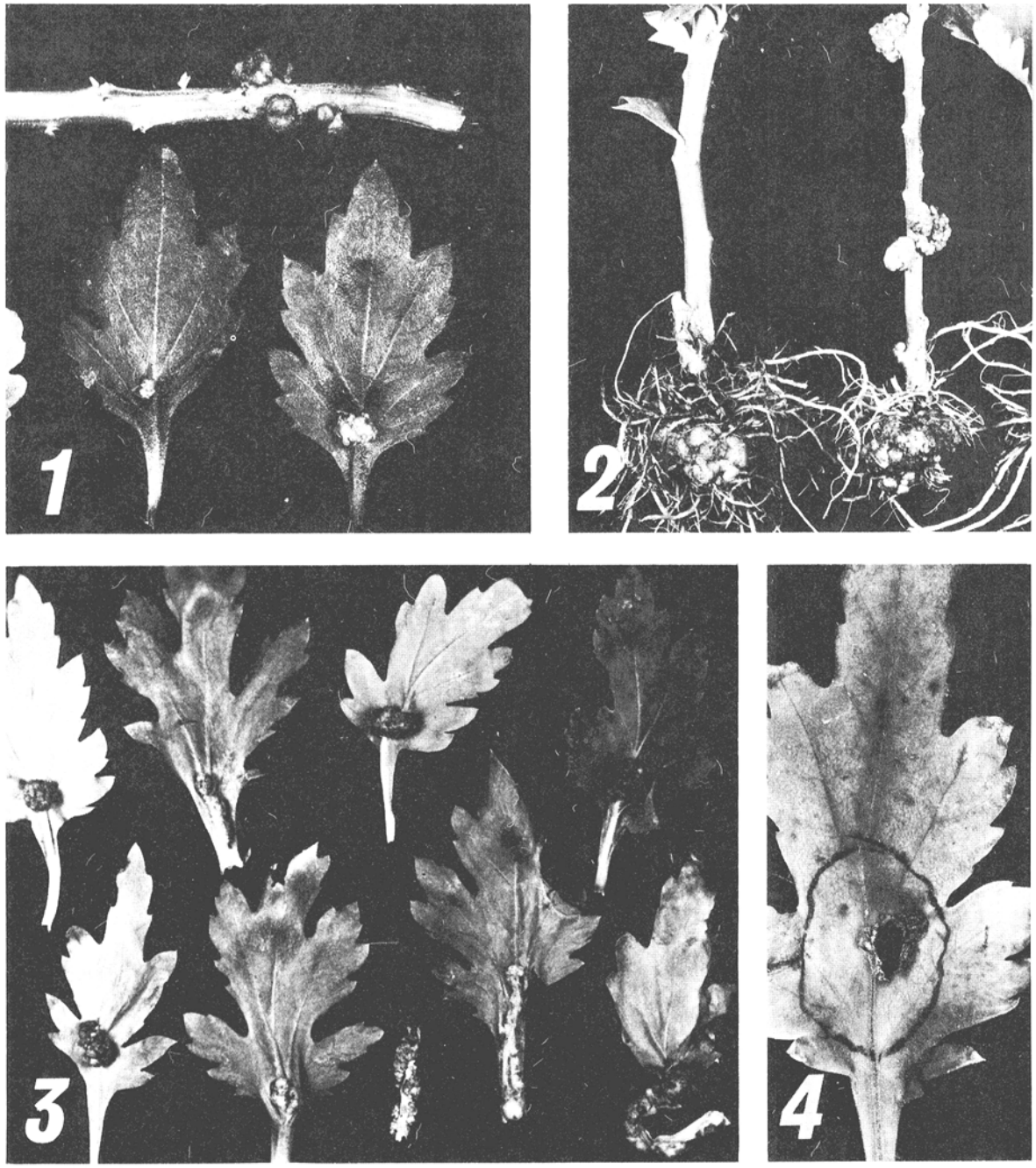


Fig. 1-4. Crown gall of chrysanthemum. **1)** Natural leaf and stem infection. **2)** Natural root infection. **3)** Leaves inoculated with *Agrobacterium tumefaciens* (Chrysanthemum strain) showing wide range of symptom expression. **4)** Inoculated leaf of highly susceptible cultivar, showing hole and necrotic tissue where gall had fallen from the leaf.

unlike the head of a cauliflower. He further states that, "if the stock is of particular value, it is safe to take stem cuttings". Kohn described the occurrence of tumors on leaf and stem of various chrysanthemum cultivars caused by *A. tumefaciens* (11).

Crown gall, caused by *Agrobacterium tumefaciens* (E. F. Smith & Towns.) Conn., 1942 (4) needs little introduction. The bacterium has a wide host range and its economic importance is well documented. In recent years, considerable work has and is being done in attempts to understand how the tumors are induced. De Ropp (7) reviewed this work up to 1951. Lippincott and Heberlein (12) in 1965 discussed the induction of leaf tumors by *A. tumefaciens*. They studied the quantitative determination of the infectivity of *A. tumefaciens* (13). Lippincott and Lippincott stated in 1969 that the attachment to a specific wound site was an essential stage in tumor initiation by *A. tumefaciens* (14). McKeen described crown gall on *Rubus*, and stated that it was systemic in its hosts and usually caused galls to form at nodes (15). Riker et al. compared bacterial plant galls and their causal agents (16).

Observations in a local, small chrysanthemum planting and preliminary laboratory work led to the research reported in this paper. Galls were observed on leaves, leaf petioles, stems, crowns, and roots of chrysanthemum. Galls were observed at terminal pinches, and at the point of lateral shoot removal. Galls frequently occurred at aboveground stem injuries. Further observations revealed the presence of basal stem or root galls on plants which exhibited galls on aboveground portions of the plants. There appeared to be a wide difference in cultivar susceptibility.

The objectives of this research were to describe clearly the symptoms on chrysanthemum caused by a crown gall bacterium, to compare the chrysanthemum isolates with other crown gall isolates, and to study the systemic action of *A. tumefaciens* in chrysanthemum. Attempts were made to verify the prolonged presence of the bacterium throughout the plants, and to show that galls were produced only if the bacteria were present at the site of an injury; and that viable bacteria could be isolated from proliferated gall tissue 4-6 weeks after gall initiation.

In previous research with *A. tumefaciens*, several workers have indicated that secondary gall formation may occur as a result of a tumor-inducing principle without the presence of viable bacteria at the site of gall formation (2, 8, 10). In this work, galls were not formed without the presence of the bacterium at an injury site. Therefore, it is postulated that the tumor-inducing principle is not essential to gall formation on chrysanthemum.

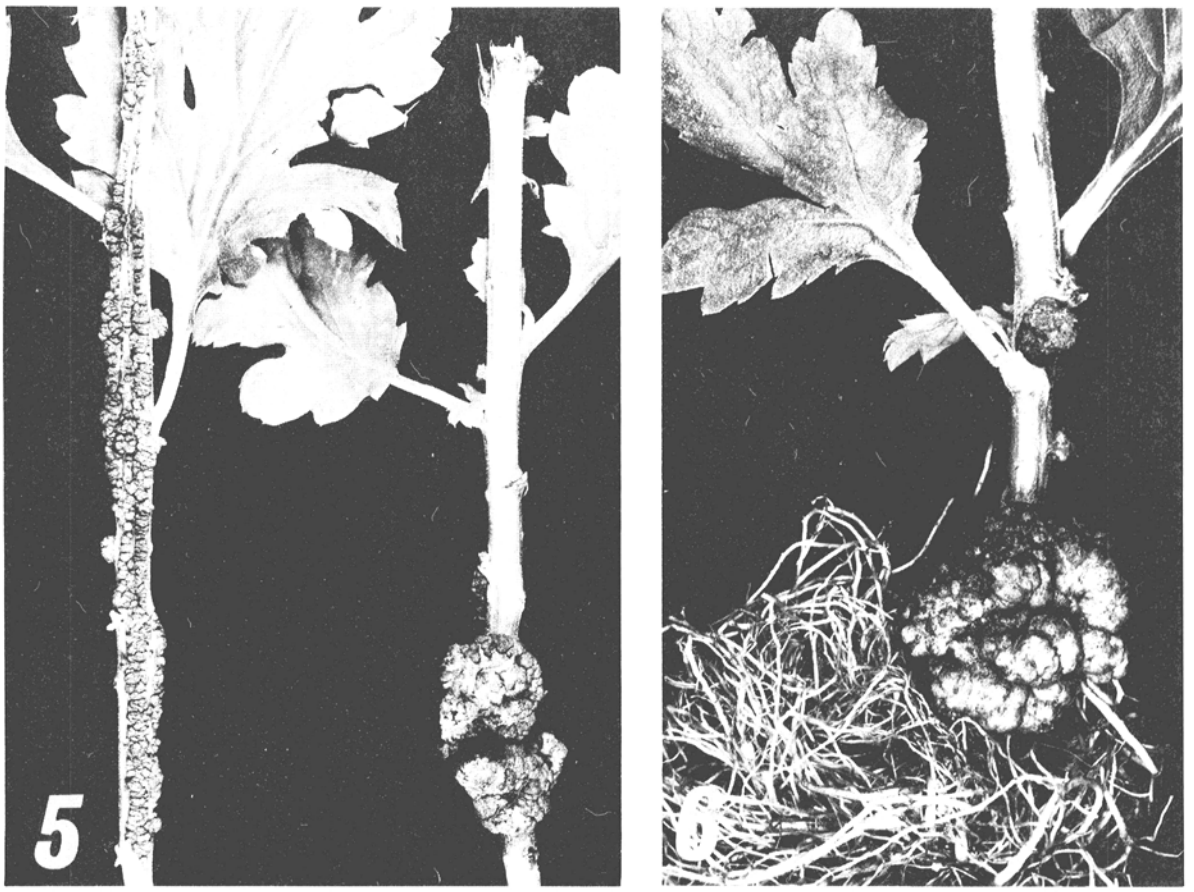


Fig. 5-6. Chrysanthemums inoculated with *Agrobacterium tumefaciens* (Chrysanthemum strain). **5)** Stem inoculation (galled stem on left resulted from stripping epidermis above point of inoculation). **6)** Root inoculation.

TABLE 1. Effect of temperature and relative humidity on gall formation caused by *Agrobacterium tumefaciens* in chrysanthemum

Average daily temperature range (C)	Average daily R.H. range (%)	Gall development
25-36	30-70	None–Few Small
18-35	50-85	Moderate
18-27	60-100	Numerous Large
25-28	60-100	Numerous Large
18-36	80-100	Numerous Very Large Proliferated

MATERIALS AND METHODS.—Indexed, rooted chrysanthemum cuttings were planted in 10.2-cm (4-inch) diameter pots in a sterilized mixture of Canadian peat, Florida field soil (fine Leon sand), and perlite (1:1:1, v/v). The plants were fertilized with a controlled-release 18-6-12 fertilizer, allowed to become established and maintained on greenhouse benches which had been thoroughly washed and rinsed with a 10% sodium hypochlorite solution.

Each pot was individually hand watered to avoid wetting the plant foliage and spattering the bacteria from one plant to another. Young, rapid-growing plants as well as mature flowering plants with hardened stems were inoculated. The greenhouses were either air-conditioned or evaporatively cooled. Inoculations were made to approximately 6,000 plants comprising 237 cultivars, ranging from highly susceptible to resistant, over a period of 10 months from December 1973 to September 1974. Temperatures in the greenhouses varied widely during this time, ranging from 18 to 36 C.

The inoculum consisted of *A. tumefaciens* type-culture (ATCC) B6 and several isolates of *A. tumefaciens* from chrysanthemum recovered from leaf galls, stem galls and root galls of naturally infected chrysanthemum. Isolates of *A. tumefaciens* type-culture B6 and *A. rubi* were obtained from the American Type Culture Collection (ATCC), Rockville, Maryland; *A. tumefaciens* from *Gypsophyla* and *Salix* were obtained from the Florida Type Culture Collection of plant pathogenic bacteria, Division of Plant Industry, Gainesville. The *Rosa, Carya,* and all other chrysanthemum isolates were obtained by the author from infected plant materials from Florida.

Inoculum was prepared in the following manner. One uniform loop from a stock culture slant on Difco nutrient agar was transferred to a nutrient agar petri plate and grown for 48 hours at ambient temperature (20-25 C). One uniform loop was transferred from the plate to 15 ml of nutrient broth, maintained for 48 hours at ambient temperatures, and then diluted by the addition of nutrient broth to give a concentration of approximately 10^8 cells per ml.

Two methods of inoculation of chrysanthemum were used with all *A. tumefaciens* isolates. Leaf, stem, and root inoculations were made with hypodermic needle injections of the nutrient broth cultures. Leaf injections were done by inserting the needle into the midvein or a lateral vein, and injecting enough of the inoculum to give a small, visibly, water-soaked area around the point of injection.

In addition, stem inoculations were made with all isolates by touching toothpicks to 48-hour nutrient agar cultures of the bacteria and inserting these into the stems of the plants.

Five plants were used as an experimental unit for each inoculation, and the tests were repeated three times. Similar groups of plants inoculated with nutrient broth were maintained as checks. Readings were made four weeks and six weeks after inoculation.

Bacterial isolations from galls occurring on leaves, stems, and roots were made by removing the galls, surface-sterilizing them for 1-3 minutes in 10% sodium hypochlorite, and rinsing them in sterile water. The galls were sectioned aseptically in sterile dishes and small sections from the inner part of the galls were placed in nutrient broth. After 48 hours, the bacteria were streaked on nutrient agar plates and *Agrobacterium* colonies were transferred to culture tubes.

All reisolates were identified as *Agrobacterium* using the methods described. In addition, 50% were reinoculated to chrysanthemum, and all induced galling.

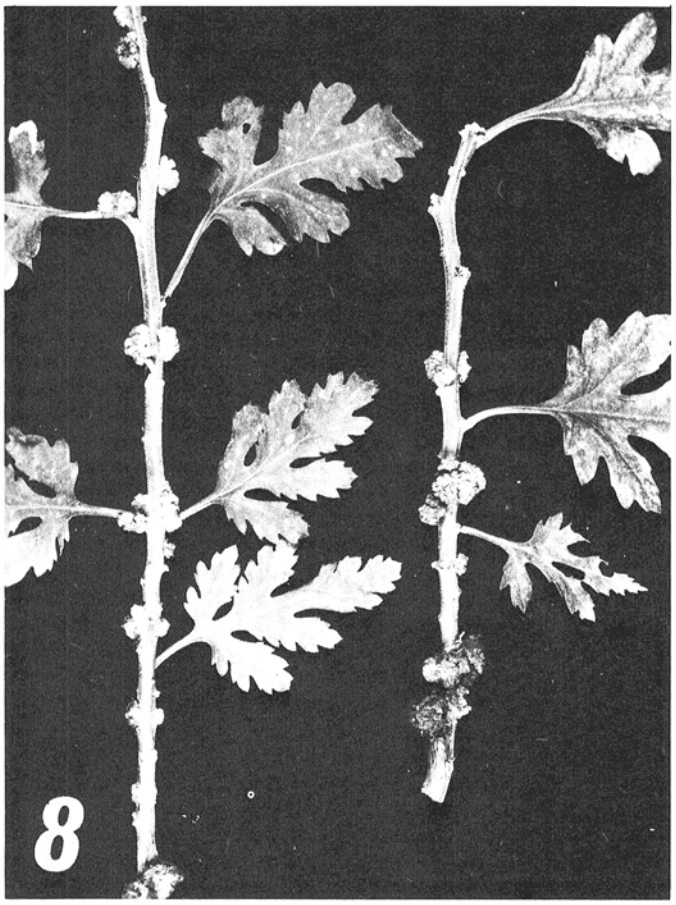
The chrysanthemum strains of *A. tumefaciens* caused galling of tomato, sunflower, and dahlia. All *A. tumefaciens* isolates induced galls on carrot slices (1), and produced cultural colonies similar to those previously described for this organism. All isolates gave positive reactions on keto-lactose agar (3).

Agrobacterium was isolated from nongalled stem areas of infected plants by removing 5-cm sections, surface-sterilizing them for 3 minutes in sodium hypochlorite, and rinsing them in sterile water. A 2-mm diameter disk was aseptically cut from the center of the stem piece and placed in nutrient broth. After 48 hours, the bacteria were streaked on nutrient agar plates and *Agrobacterium* colonies recovered.

RESULTS.—*Symptomatology.*—Symptoms of the disease from natural infection appeared on the lateral or midveins of the leaves as smooth, light tan-colored galls 2-4 mm in diameter. Galled tissue appeared on both sides of the leaves from the point of infection. Naturally occurring stem galls appeared at points of injury, terminal pinches, and points of lateral shoot removal (Fig. 1). Stem and crown galls were typical, varying in size from 2-4 cm. Basal, below ground, stem, and root galls were typical, and reached diameters of 4-5 cm (Fig. 2). *A. tumefaciens* was isolated from the naturally occurring galls.

Inoculation of susceptible chrysanthemum leaves with the chrysanthemum strain of *A. tumefaciens* resulted in galls of varying size, texture and ramification through the leaf veins (Fig. 3). Large galls developed from leaf inoculations on some highly susceptible cultivars, later dropping from the leaves leaving a hole and necrotic tissue around the original galled area (Fig. 4). Stem and root inoculations on susceptible cultivars resulted in typical gall formation. Galls resulting from inoculations generally grew two to three times larger than those caused by natural infections (Fig. 5-6).

Under the conditions of these tests, small galls were visible on all plant parts 7-10 days after inoculation. Maximum gall development occurred 6-8 weeks after inoculation. Temperatures, within the range used, had less effect on gall development than did humidity. Gall development was most rapid at a temperature range of 18-36 C with 80-100% relative humidity (RH). Poor and



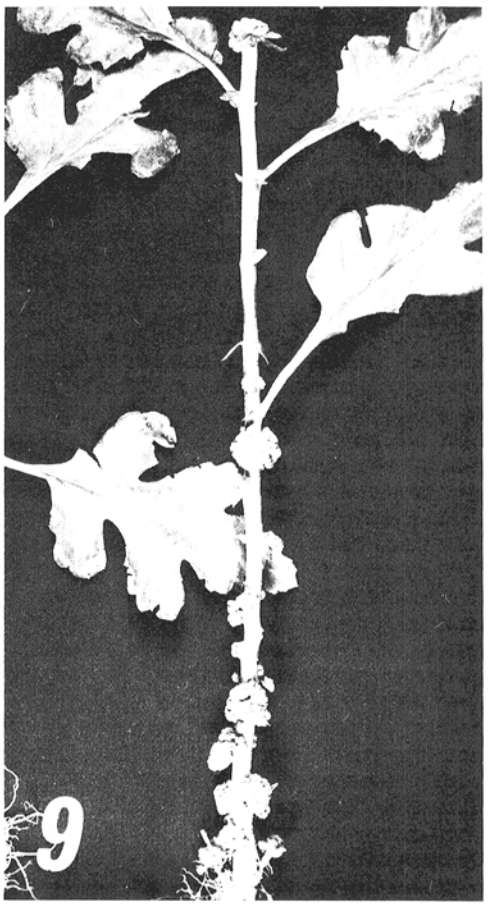


Fig. 7-9. Susceptible and resistant cultivars. Plants showing systemic nature of the bacteriu. **7)** Chrysanthemum cultivar, Deep Ridge; plant on left inoculated with chrysanthemum strain of *Agrobacterium tumefaciens.* Plant on right inoculated with B6 strain. **8, 9)** Chrysanthemum plants inoculated with *Agrobacterium tumefaciens* near base of stems. Subsequent gall development at tip pinches, sterile needle punctures, and leaf or lateral shoot removal.

delayed development occurred at 25-36 C with 30-70% RH (Table 1) (6, 18).

Inoculation with other isolates of Agrobacterium tumefaciens.—The crown gall bacteria obtained from *Rosa, Salix, Carya, Gypsophyla,* and *Rubus (Agrobacterium rubi)* did not produce galls on

chrysanthemum. Isolate B6 was less pathogenic to chrysanthemum, affecting 50% of the 208 chrysanthemum cultivars which were affected by the chrysanthemum isolates (Fig. 7). However, isolate B6 affected a few cultivars which were resistant to the chrysanthemum isolates. Of all the cultivars which were inoculated, 10% were resistant to all *A. tumefaciens* isolates used. No galling occurred on leaves, stems, or roots regardless of the method of inoculation or environmental conditions. An average of 15 plants were inoculated for each cultivar in separate tests. Some plants were held for two months to rule out the possibility of delayed gall development.

Systemic nature of Agrobacterium tumefaciens in chrysanthemum.—From field observations and inoculations, it appeared likely that the bacterium moved systemically within the plants. Three leaf injections and two stem injections on each of the 15 plants used per cultivar were made with the B6 strain and chrysanthemum isolates on disease-free, greenhouse-grown plants into the lower leaves and stems near the ground line. After gall initiation had occurred, sterile needle punctures and terminal pinches were made and lateral shoots removed from points of inoculation to the plant tips. Tips and shoots were removed by clipping with sterilized scissors. After 2-4 weeks, gall formation had occurred at approximately 85% of the injured locations (Fig. 8, 9). When it was attempted, the bacterium was reisolated from all galled tissue. In all reisolations from inoculated plants, the bacterium was identified by cultural colony characteristics, gall production on carrot slices, a positive reaction on keto-lactose agar, and by gall formation when reinoculated in chrysanthemum.

To further establish the translocation of *A. tumefaciens* through the stems of chrysanthemum, isolations were made from uninjured, nongalled stems at distances of 24-44 cm above the area of basal inoculations and gall formation. Bacteria were recovered from the vascular tissue of the stem, and cross sections revealed their presence in large numbers. It was further demonstrated that the bacteria also moved downward through the stems. After tip inoculations and gall formation, bacteria were recovered from the stems at varying distances below the point of inoculation. *A. tumefaciens* (B6 and chrysanthemum isolates) was systemic in all susceptible chrysanthemum cultivars checked irrespective of the degree of susceptibility. The number and size of galls formed at sites of injury were consistent with the pattern of gall formation on any specific cultivar relative to its degree of susceptibility.

Hardened stems on flowering plants were inoculated with a hypodermic needle to determine if galls would be produced on older tissue. Seventy-five percent of inoculations made in susceptible cultivars resulted in typical gall formation. There was usually a time lag before gall initiation was observed. However, galls developed to the same size and extent as those on rapidly expanding internodes on younger plants. Inoculations by injection of bacterial suspensions into older mature leaves resulted in typical gall formation. *A. tumefaciens* was readily isolated from galls on older stems and from large, hardened root galls. Viable bacteria were recovered repeatedly from 6- to 8-week-old galls.

DISCUSSION.—Results of these studies indicate that gall formation on chrysanthemum caused by *A. tumefaciens* occurs at the site of an injury, only if the bacterium is present.

It was demonstrated that the bacteria remained viable in galled tissue and within the plant for periods of at least 6-8 weeks. The bacterium was systemic within the plant stems and leaf veins, vascular tissue-specific, and it moved both up and down the stems from the point of inoculation.

This systemic nature of *A. tumefaciens* in chrysanthemum is of vital importance to growers, because inoculum can be spread from one plant to another through terminal pinching and lateral disbudding operations, which are common procedures in commercial chrysanthemum production. In this work, repeated terminal pinching of chrysanthemum plants having only below-ground root and basal stem galls resulted in galls being formed at the point of the pinch. Galls were frequently formed at disbudding or injury sites on older plants at distances of up to 44 cm from the point of infection. All galls on chrysanthemum appeared to be primary galls because of their nature and development, the demonstrated presence of the bacterium in the plant, and the reisolation of the pathogen.

Under the variable greenhouse conditions used, temperatures within the range recorded had less effect on gall development than RH. Most rapid gall development occurred at 18-36 C with 80-100% RH.

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