Systemic Movement of *Agrobacterium tumefaciens* in Symptomless Stem Tissue of *Chrysanthemum morifolium*

J. B. JONES, IFAS, University of Florida, Gulf Coast Research and Education Center, 5007 60th Street East, Bradenton 34203, and B. C. RAJU, Weyerhaeuser Tissue Culture Laboratory, 4051 Fudge Road, Apopka, FL 32703

ABSTRACT

Jones, J. B., and Raju, B. C. 1988. Systemic movement of *Agrobacterium tumefaciens* in symptomless stem tissue of *Chrysanthemum morifolium*. Plant Disease 72:51-54.

Agrobacterium tumefaciens was isolated infrequently from apparently healthy stem tissue distal to developing galls. The incidence of recovery of *A. tumefaciens* ranged from 0 to 80% from stems of different cultivars 2 wk after inoculation. The bacterium was isolated from only 11.4% of the 377 stems assayed. Wounds were made aseptically above the developing galls, and gall development occurred in 2.2% of the 1,400 sites. In one study, when stems of six cultivars were wounded, galls developed on stems of only three of the cultivars. The percentages of inoculated stems that developed galls were 44, 14, and 2% on the cultivars Dark Yellow Paragon, Florida Marble, and Circus, respectively. In a second study, the incidence of gall development at wound sites was 4% on Matador stems wounded 1 day after inoculation. However, gall formation was not detected on stems of Matador plants wounded 0, 3, or 7 days after inoculation or at any of the wound sites on Circus Bronze stems that were wounded at the same time periods. When cuttings were taken from inoculated Surf and Circus stock plants, the incidence of gall development ranged from 0 to 60% on the cut ends of the stock plants and from 0 to 40% on the bases of the cuttings. Generally, gall formation was not induced on the wound surface of the stock plant (4.4% of 160 wound surfaces developed galls) or the cutting (3.1% of 160 cuttings developed galls) when cuttings were taken at locations on the stem above developing galls.

Agrobacterium tumefaciens has been reported to be systemic in plant tissue (1,5,6). In *Chrysanthemum morifolium* Ramat., *A. tumefaciens* was previously observed to readily move systemically through the stem to points distal to developing galls (6). Also, gall formation readily occurred at points on the stem above the developing gall where wounds were made with sterilized needles (6). Clarification of the systemic nature of *A. tumefaciens* and of the degree of gall formation on healthy plant parts above a developed gall is important because gall development has not been observed by us to be common at locations distal to the gall. A preliminary report was published (3).

The objective of this study was to reevaluate the degree of systemic movement of *A. tumefaciens* in chrysanthemum stems and the development of secondary galls at wound sites above developing galls.

Florida Agricultural Experiment Stations Journal Series No. 7395.

Accepted for publication 2 September 1987.

MATERIALS AND METHODS

Bacterial strains. The virulent strains of *A. tumefaciens* that were isolated from chrysanthemum were supplied by J. W. Miller (Division of Plant Industry, Gainesville) and R. E. Stall (Department of Plant Pathology, University of Florida, Gainesville). The strains were maintained in sterile tap water for long-term storage. Inoculum was produced by growing the bacterium for 24–48 hr at 28 C on nutrient-yeast-dextrose agar (NYDA) (2). One strain, designated 077, was selected for resistance to rifampicin and nalidixic acid as described previously (9) by incorporating both antibiotics into NYDA at 100 μg/ml. The recovery efficiency of the antibiotic mutant was over 100% on NYDA amended with the two antibiotics. The antibiotic markers did not appear to affect the virulence of the selected strain.

Plant culture. Rooted chrysanthemum cuttings were planted in an artificial medium consisting of Florida peat, sand, vermiculite, and perlite (5:3:3:1). Calcium carbonate, dolomitic lime, hydrated lime, superphosphate, and Micromax (Sierra Chemical Co., Milpitas, CA), a minor element supplement, were applied at 5.54, 5.54, 0.549, 1.39, and 0.83 kg/m^3, respectively. The plants were grown under greenhouse conditions (18–30 C and 70–100% relative humidity) and were fertilized weekly with Nutrileaf (Hanover, PA) at the rate of 3.6 g/L.

Inoculation procedure. Sterilized

toothpicks dipped in a mass of bacteria were inserted through the entire diameter of the base of young developing stems (8).

Cuttings and wounds as sites of secondary gall formation. At various periods after inoculation, cuttings were taken from recently matured tissue and planted in individual 10-cm pots containing the artificial medium described. The plants were watered as required. About 4–6 wk after harvesting the cuttings, the bases of the cuttings and stock plants were checked for gall formation at the wound site created by the cutting.

Artificial wound sites were created by piercing the stem with sterile pins so that the pin penetrated completely through the diameter of the stem. The wounds began 20 cm above the inoculation point and extended down the stem at 1-cm intervals. Wounding ceased 1 cm above the inoculation point. The wound sites were checked for gall formation 4–6 wk later.

Isolation. To determine if *A. tumefaciens* were present in nonsymptomatic stem tissue above the inoculation point, 5-mm stem sections were surface-sterilized in 0.535% sodium hypochlorite for 5 min, rinsed in sterile deionized water, and crushed in sterile deionized water. The suspension was incubated 30 min, and loopfuls of the suspension were streaked onto NYDA. In the second method of isolation, 20-cm-long stem segments were collected from plants that had been inoculated with the antibiotic-resistant mutant. The stems were excised 1 cm above the inoculation site. The foliage was removed, and the stem was surface-sterilized in 0.525% sodium hypochlorite for 5 min, then rinsed three times in sterile deionized water and cut into 20 1-cm sections. Each of the 20 sections was placed in 40 ml of sterile nutrient broth in 125-ml flasks. The flasks were placed on a rotary shaker and shaken at 75 rpm. After 3, 6, 12, and 24 hr, 0.1-ml samples of the broth were placed on NYDA amended with rifampicin and nalidixic acid. The plates were incubated at 28 C for 72 hr and checked for growth characteristic of *A. tumefaciens*. All suspect colonies were either tested for pathogenicity on Surf chrysanthemum stems or on tomato stems.

Studies with the wild-type strains of *A. tumefaciens*. In the first study, which involved two tests, Surf and Circus chrysanthemum plants were inoculated with the wild strain of *A. tumefaciens* or wounded with sterilized toothpicks (control) and grown in the greenhouse. A mat irrigation system was used to eliminate any possible aerial spread of the bacterium by splashing water. Cuttings were taken from each of five control and five inoculated plants 1, 2, and 4 wk after inoculation. A 5-mm cross section was excised from the base of each cutting and assayed for *A. tumefaciens* in deionized water. The cuttings were rooted and checked for crown gall development after about 6 wk.

Surf and Circus cuttings in two tests were inoculated with *A. tumefaciens* as previously described and sampled 7, 14, and 28 days later. Five plants of each cultivar were sampled at each date. The stems were cut about 1–2 cm above the inoculation site, and chips (about 0.5 cm) were removed from the base of each cutting. The chips were assayed for *A. tumefaciens*. In a final attempt to detect the bacterium, chips from the five plants for each sampling date were bulked and assayed for *A. tumefaciens* 44 and 39 days after inoculation in tests 1 and 2, respectively.

In a study where five strains of *A. tumefaciens* supplied by R. E. Stall were inoculated (one strain per plant) into the bases of Circus stems, five cuttings were sampled 2 and 4 wk after inoculation with each strain. They were rooted, and both the rooted cuttings and the stems on the stock plants were checked for gall development after about 42 days.

Studies with the antibiotic mutant strain of *A. tumefaciens*. The previous studies were inconclusive for determining the occurrence of the bacterium in healthy stem tissue above the galls on the stem. Thus, the antibiotic-resistant mutant described earlier was used for more critical studies on detection of low populations of *A. tumefaciens* in apparently healthy stem tissue. The purpose was to enrich *A. tumefaciens* in nutrient broth to make detection more likely.

Cuttings of the chrysanthemum cultivars Dark Yellow Paragon, Comet Snow, Bright Yellow Mary Shoesmith, Florida Marble, Improved Mefo, Circus, and Iceberg were grown for 3 wk and then inoculated with a strain of the antibiotic-resistant mutant *A. tumefaciens*. Two weeks after inoculation, five inoculated stems were assayed for *A. tumefaciens* in nutrient broth.

Plants of chrysanthemum cultivars Improved Mefo, Florida Marble, Dark Yellow Paragon, Iceberg, Bright Yellow Mary Shoesmith, and Circus were inoculated with the antibiotic mutant at least 20 cm beneath the growing point. The inoculated plants were then wounded with sterile pins immediately after inoculation. Four weeks after inoculation, the stems with wounds above the inoculation site were rated for gall formation. Two and 4 wk after inoculation, healthy-appearing stems on inoculated plants without wounds above the inoculation site were harvested 1–2 cm above the inoculation site and assayed for *A. tumefaciens* using nutrient broth enrichment as described previously.

Matador and Circus Bronze cuttings were grown for 3 wk. They were then wounded with sterile pins 0, 1, 3, and 7 days after inoculation with the antibiotic-resistant mutant of *A. tumefaciens*. The experiment consisted of five replicates for each treatment. After 8 wk, the wound sites were checked for gall development. Three, 7, and 14 days after inoculation, 5-mm sections were taken 1–2 cm above the inoculation site and checked for the bacterium in sterile, deionized water. The macerated tissue then was assayed for *A. tumefaciens* using nutrient broth enrichment.

Stems of Circus, Surf, Ritz, Garland, and Florida Marble were inoculated separately with two wild-type strains and the 077 strain with select antibiotic resistance. Cuttings were taken 2 and 4 wk after inoculation. The cuttings and

Table 1. Crown gall development at the wound site of cuttings and on the resultant wound site on the stock plant of two cultivars of chrysanthemum when cuttings are taken from chrysanthemum plants above actively developing crown galls

	Test 1				Test 2			
	Circus		Surf		Circus		Surf	
Treatment	Stock plant[a]	Cutting[b]	Stock plant	Cutting	Stock plant	Cutting	Stock plant	Cutting
Inoculated								
Cuttings taken after 1 wk[c]	0[d]	0	1	1	0	0	1	2
Cuttings taken after 2 wk	0	0	3	0	0	0	0	0
Cuttings taken after 4 wk	0	0	0	0	0	0	0	0

[a] Galls that developed at point on stock plant 6 wk after harvesting cuttings from stock plants previously inoculated with *Agrobacterium tumefaciens*.
[b] Galls that developed at base of cutting 6 wk after harvesting cuttings from inoculated stock plant.
[c] Weeks after inoculation in which cuttings were taken.
[d] Represents the number of replicates of five where galls were observed.

stock plants were grown for 6 wk more and observed for gall development.

RESULTS

Crown gall formation was noticeable 2 wk after inoculation. All cultivars tested developed stem galls at the inoculation sites. Isolation of the bacterium from healthy-appearing stem tissue was low in frequency. Thus, quantification of the bacterium as to concentration in stem pieces was not possible.

Studies with the wild-type strains. Gall development occurred sporadically on the stock plants where cuttings were taken 1 and 2 wk after inoculation (Table 1). Galls were only observed on Surf stock plants at the site of the cutting and also on the cuttings. The bacterium was not isolated from any of the assayed chips. One gall was observed on a control stock plant from which a cutting was taken 2 wk after inoculation.

The bacterium was not detected in symptomless Surf and Circus stem tissue 7, 14, or 28 days after inoculation in two tests. However, when stem samples were bulked together at the end of the experiment and assayed for the bacterium, *A. tumefaciens* was recovered from nonsymptomatic stems at 44 days in test 1 and at 39 days in test 2.

Of 25 cuttings taken 2 wk after inoculation, two cuttings developed galls at the bases, whereas none of the cuttings harvested 4 wk after inoculation developed galls. Only one of 25 wound sites of the stock plant from which cuttings were taken 2 wk after inoculation developed a gall. None of the 25 wound surfaces on the stock plant developed galls when cuttings were taken 4 wk after inoculation.

Studies with the antibiotic-resistant mutant of *A. tumefaciens*. Seven cultivars of chrysanthemum were compared for systemic movement of *A. tumefaciens*. The bacterium was recovered from 13 of 35 stems 14 days after inoculation (Table 2). When samples were taken 25 and 42 days after inoculation, *A. tumefaciens* was recovered from one and seven of 35 stems, respectively.

Galling occurred above the inoculation sites in the wound sites of inoculated Circus, Dark Yellow Paragon, and Florida Marble stems, respectively (Table 3). The bacterium was isolated from a low percentage of the stems on the two sampling dates.

Galls formed at two of 100 wound sites of Matador stems wounded 1 day after inoculation but not on stems of either cultivar wounded 7 days after inoculation. The bacterium was isolated from three Matador stems 3 days after inoculation and from one Circus Bronze stem 14 days after inoculation. The bacterium was not detected in symptomless stem tissue 7 days after inoculation.

In a second test with five cultivars, gall development occurred infrequently at the wound site created by the cutting (on only one Florida Marble stock plant) and was not observed at the base of any cutting. A 5-mm section from the base of the cutting was assayed for the bacterium on NYDA and NYDA amended with nalidixic acid and rifampicin, but the bacterium was not detected.

DISCUSSION

A. tumefaciens has been reported to be readily isolated from symptomless tissue of *C. morifolium* (6). Miller (6) noted large numbers of the bacterium in the vascular tissue of nongalled stems at distances 24–44 cm above the area of basal galls. Using conventional plating techniques for isolation of wild-type strains and an enrichment procedure with a selected antibiotic mutant for more selective isolation of *A. tumefaciens*, we were able to periodically isolate the bacterium from apparently healthy stem tissue. However, the frequency of isolation from symptomless tissue was on the average less than 10%, indicating that the internal populations in the vascular tissue were not as high as Miller (6) observed.

Miller (6) also noted that if stem tissue above the galls was wounded, a high percentage of the wounds developed galls. This was further proof that the bacterium was systemic and that gall formation was readily induced on nonsymptomatic tissue. Our studies were less favorable for demonstrating the capacity of the bacterium to cause galling at locations above the galled tissue since galls developed on less than 10% of the wound sites above the inoculation point. Also, gall development was infrequent on the base of the cutting and at the cutting wound on the stock plant.

There are plausible explanations for the discrepancies in results. First, the strains used by Miller (6) were not the same as those used in our study. Second, the cultivars used in this study probably were not the same as Miller's (6). One important fact that our study emphasizes is that there are pronounced differences among cultivars in terms of recovery of *A. tumefaciens* from nonsymptomatic tissue. Also, the populations of *A. tumefaciens* in nonsymptomatic tissue are extremely low. Finally, the sampling date appears to be critical with regard to recovery of the bacterium.

The fact that *A. tumefaciens* appears to be systemically translocated at extremely low levels supports the idea that gall development on cuttings taken from apparently healthy tissue at points above obviously diseased or galled tissue is unlikely when proper surface disinfestation procedures are followed. The recovery of *A. tumefaciens* at low levels from all cultivars helps explain the low level of gall development induced at wound sites distal to the inoculation site. Although systemic movement does appear to be a factor in secondary gall development, a high proportion of apparently healthy plants may be produced from cuttings taken from plants containing galled tissue. Thus,

Table 2. Recovery of a rifampicin-nalidixic acid mutant strain of *Agrobacterium tumefaciens* from chrysanthemum stems that were sampled above actively developing crown galls

Cultivar	Number of stems from which *A. tumefaciens* was isolated[a]		
	14[b]	25	42
Florida Marble	1[c]	0	1
Improved Mefo	0	0	0
Circus	2	0	1
Comet Snow	3	0	0
Dark Yellow Paragon	4	0	1
Bright Yellow Mary Shoesmith	2	0	1
Iceberg	1	1	3

[a] Inoculation was achieved by inserting a sterile toothpick dipped in *A. tumefaciens* into stems of recently planted cuttings.
[b] Number of days after inoculation.
[c] Number of replicates of five that were positive for isolation of *A. tumefaciens*.

Table 3. Recovery of an antibiotic-induced mutant of *Agrobacterium tumefaciens* from symptomless chrysanthemum stems and induction of galls at wound sites on symptomless chrysanthemum stems above actively developing crown galls

Cultivar	Number of stems from which *A. tumefaciens* was isolated		Wound sites that developed galls above actively developing galls
	14 Days after inoculation	22 Days after inoculation	
Improved Mefo	0[a]	0	0[b]
Florida Marble	2	2	22
Dark Yellow Paragon	0	2	7
Iceberg	0	0	0
Bright Yellow Mary Shoesmith	0	0	0
Circus	0	0	1

[a] Number of stems of five from which *A. tumefaciens* was isolated.
[b] Number of artificial wound sites of 100 that developed galls. Wound sites were created by inserting sterile pins in the stem above the inoculation site the day of inoculation.

reduction in gall development on *C. morifolium* may be feasible by appropriate sanitation in addition to chemical (7) or biological control (4,7).

LITERATURE CITED

1. Burr, T. J., and Katz, B. H. 1983. Isolation of *Agrobacterium tumefaciens* biovar 3 from grapevine galls and sap, and from vineyard soil. Phytopathology 73:163-165.
2. Jones, J. B., McCarter, S. M., and Gitaitis, R. D. 1981. Association of *Pseudomonas syringae* pv. *syringae* with a leaf spot disease of tomato transplants in southern Georgia. Phytopathology 71:1281-1285.
3. Jones, J. B., and Raju, B. C. 1984. Systemicity of *Agrobacterium radiobacter* pv. *tumefaciens* and low frequency of galling on chrysanthemum cuttings. (Abstr.) Phytopathology 74:882.
4. Kerr, A., and Htay, A. 1974. Biological control of crown gall through bacteriocin production. Physiol. Plant Pathol. 4:37-44.
5. Lehoczky, J. 1968. Spread of *Agrobacterium tumefaciens* in the vessels of the grapevine, after natural infection. Phytopathol. Z. 63:239-246.
6. Miller, H. N. 1975. Leaf, stem, crown, and root galls induced in chrysanthemum by *Agrobacterium tumefaciens*. Phytopathology 65:805-811.
7. Miller, H. N., and Miller, J. W. 1976. Control of crown gall on chrysanthemum with a nonpathogenic bacterium and with selected antibiotics. Proc. Fla. State Hortic. Soc. 89:301-302.
8. Miller, H., Miller, J. W., and Crange, G. L. 1975. Relative susceptibility of chrysanthemum cultivars to *Agrobacterium tumefaciens*. Plant Dis. Rep. 59:576-581.
9. Weller, D. M., and Saettler, A. W. 1978. Rifampicin-resistant *Xanthomonas phaseoli* var. *fuscans* and *Xanthomonas phaseoli*: Tools for field study of bean blight bacteria. Phytopathology 68:778-781.