Effect of Temperature, Plant Age, and Infection Site on the Severity of Crown Gall Disease in Radish

L. W. Moore and David T. Tingey

Assistant Professor, Department of Botany and Plant Pathology, Oregon State University; and Plant Physiologist with the Environmental Protection Agency, Corvallis, Oregon 97331, respectively.

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ABSTRACT

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Radish growth was retarded by crown gall as measured by leaf area, dry weight, and the rate of leaf initiation (Plastochron Index). The largest growth reduction from crown gall was temperature-dependent and occurred at the same temperatures that maximized top growth of noninfected control plants (day/night temperatures of 30/26 and 34/30 C). Crown gall had little effect on plant growth at 14/10 C. Growth retardation at 30/26 and 34/30 C begins within 2 days after inoculation as shown by the Plastochron Index. Significant differences in plant growth occurred when various sites on plants were infected. Total dry weight reduction was greatest when the upper hypocotyl was infected. Growth reduction was most severe when younger plants were inoculated regardless of the infection site. Young plants infected in the upper hypocotyl were permanently stunted, and failed to recover after an extended growth period.

Additional key words: Agrobacterium tumefaciens.

The growth changes of galled *Prunus* cultivars resulting from natural infection with *Agrobacterium tumefaciens* vary from no visible effect to severe stunting and sometimes death. Symptom variability has resulted in conflicting reports on the severity of this disease. Some studies suggest that crown gall seriously affects tree growth and crop production (5, 7), and others suggest that it does not (9). Early work generally lacks quantitative measurements of host response.

The variable host response to crown gall disease appears to be related to the site of infection (5, 7), age of the tree when infected, and the environmental conditions after inoculation. Radish plants were chosen as a model system to study these variables because they germinate rapidly and uniformly, mature quickly, and growth of the plant parts can be measured easily. The work reported here was designed to: (i) quantify the growth of radish plants infected with *Agrobacterium tumefaciens,* (ii) determine the importance of different inoculation sites relative to subsequent plant damage, and (iii) determine the influence of temperature on the growth of infected plants.

MATERIALS AND METHODS

Radish (*Raphanus sativus* L. 'Cherry Belle') seeds screened for uniform size and color were planted in Jiffy-Plus (a commercial soil mix containing a balanced, slow-release fertilizer) in 225-cc styrofoam cups (with bottom drainage) and covered with white silica sand. The cups were placed immediately under a 12-hour photoperiod (15,064 lx) at 30 C night and 26 C day in controlled environment chambers (42-46% relative humidity). Four days after seeding, the chambers were adjusted to day/night temperatures of 14/10, 22/18, 30/26, 34/30, and 38/34 C, and the plants were inoculated 2 days later during the first 3 hours of the photoperiod, unless specified otherwise. The photo- and thermal periods were 12 hours long. Plants were watered daily with sufficient distilled water to drip through the bottom drainage holes.

Plant inoculation sites included the petioles of both cotyledons, the upper part of the hypocotyl, and the transition zone between the lower part of the hypocotyl and the tap root. A 3-ketolactose-negative strain of *A. tumefaciens* Conn, 1942, strain B-234 (initially isolated from a naturally galled peach tree by J. DeVay), was selected for this study because it was the most virulent of 10 isolates examined by preliminary screening on radish plants. Inoculum of strain B-234 was prepared by suspending the bacteria in distilled water. The bacteria were cultured for 24-48 hours on potato-dextrose agar containing 0.5% $CaCO_3$ before use. The inoculum was standardized to about 7×10^8 colony-forming units/ml. Six plants for each treatment were inoculated by passing a 0.51-mm diameter (25-gauge) hypodermic needle completely through the inoculation site while injecting the bacterial suspension. Control plants consisted of noninjected plants because preliminary work showed there was no significant difference in growth between noninjected control plants and those injected with sterile water.

The effect of crown gall on plant growth was determined by dry weight measurements. Plant tops, roots, and gall tissue were weighed separately after being dried at 70 C for 1 week. Tumor weight often contributed significantly to the total plant weight, masking an obvious visual reduction in overall plant size. Tumors could be dissected cleanly from infected cotyledons and weighed

separately, but the demarcation between tumor and host tissue in the infected hypocotyl was not distinct, and removal of the tumors was subjective. Consequently, total leaf area was measured photometrically with a type-AAM Hayashi Denko automatic area meter as an additional estimate of plant growth.

The methods described above provided data about the effect of crown gall on plants 22 days after inoculation, but provided no indication of when infection began to influence plant growth. The Plastochron Index (PI) developed by Erickson and Michelini (3) was used to provide a continuous estimate of growth from days 8 to 28 as measured by the rate of leaf initiation. This index is based on the time interval between initiation of two successive leaves and is calculated from the following equation:

$$PI = n + \frac{\log L_n - \log 10}{\log L_n - \log L_{n+1}}$$

Data for the equation are obtained by counting the leaves longer than 10 mm (n) and measuring the lengths of the leaf just longer (L_n) and the leaf just shorter (L_{n+1}) than 10 mm. Measurements were made every other day, the PI was plotted against time, and the rate of leaf initiation was calculated.

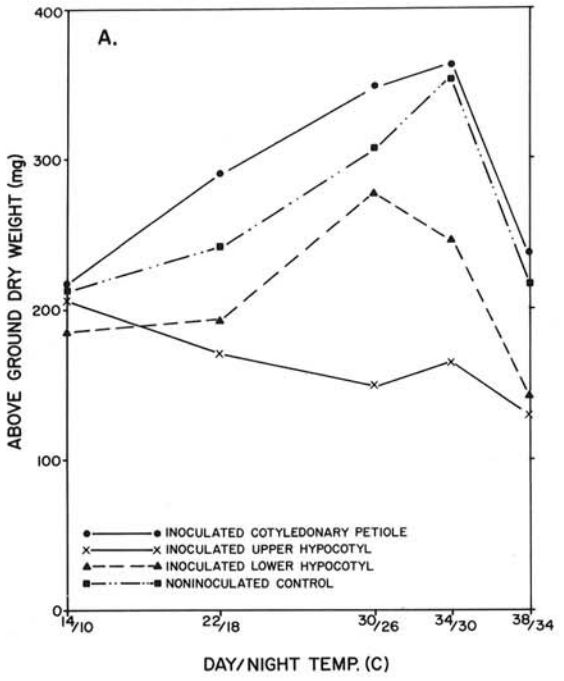
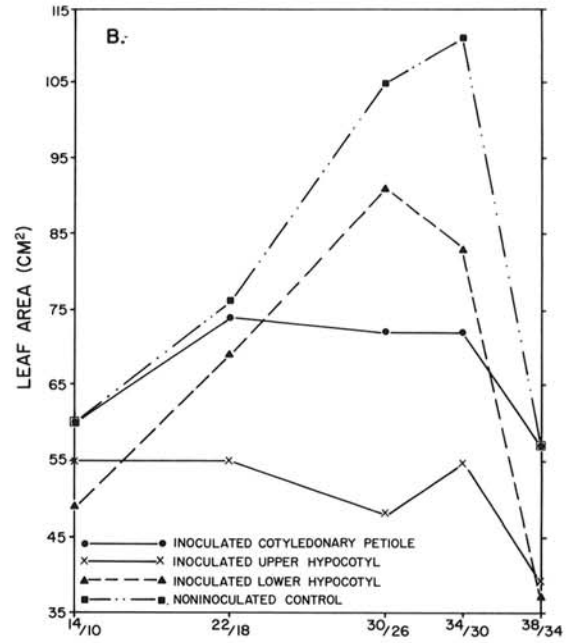
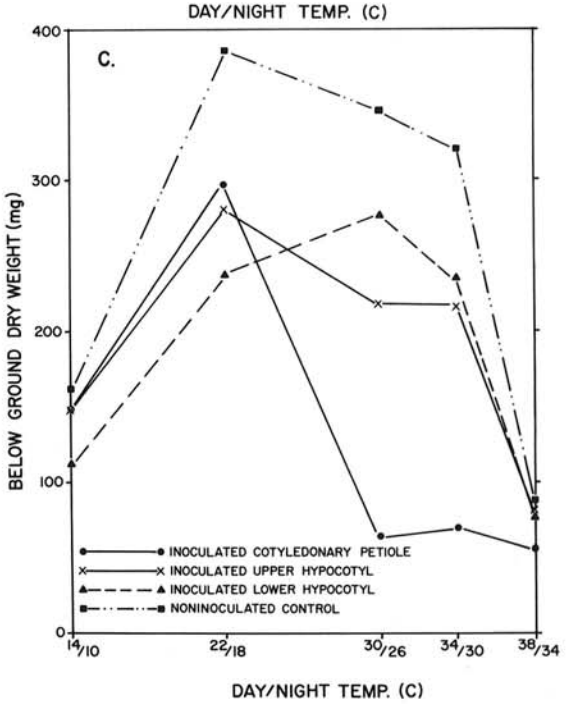


Fig. 1-(A to C). Influence of various day/night temperatures, infection by *Agrobacterium tumefaciens*, and site of infection on growth of radish plants. Each point is the mean of six observations. **A)** Dry weight of aboveground tissues; including gall tissue associated with inoculated cotyledonary petioles. $S\bar{x}$ = 21 mg. **B)** Total leaf area. $S\bar{x}$ = 6 cm^2. **C)** Dry weight of belowground tissues; including gall tissue associated with the hypocotyl inoculation sites. $S\bar{x}$ = 31 mg.

RESULTS

Effect of temperature on above- and belowground growth of variously inoculated radish plants.—The weight of leaves and shoots of the control plants harvested 28 days after seeding increased continuously to a maximum as the day/night temperatures increased from 14/10 to 34/30 C (Fig. 1-A). In contrast, the corresponding weight of plants infected in the upper hypocotyl decreased significantly over the entire





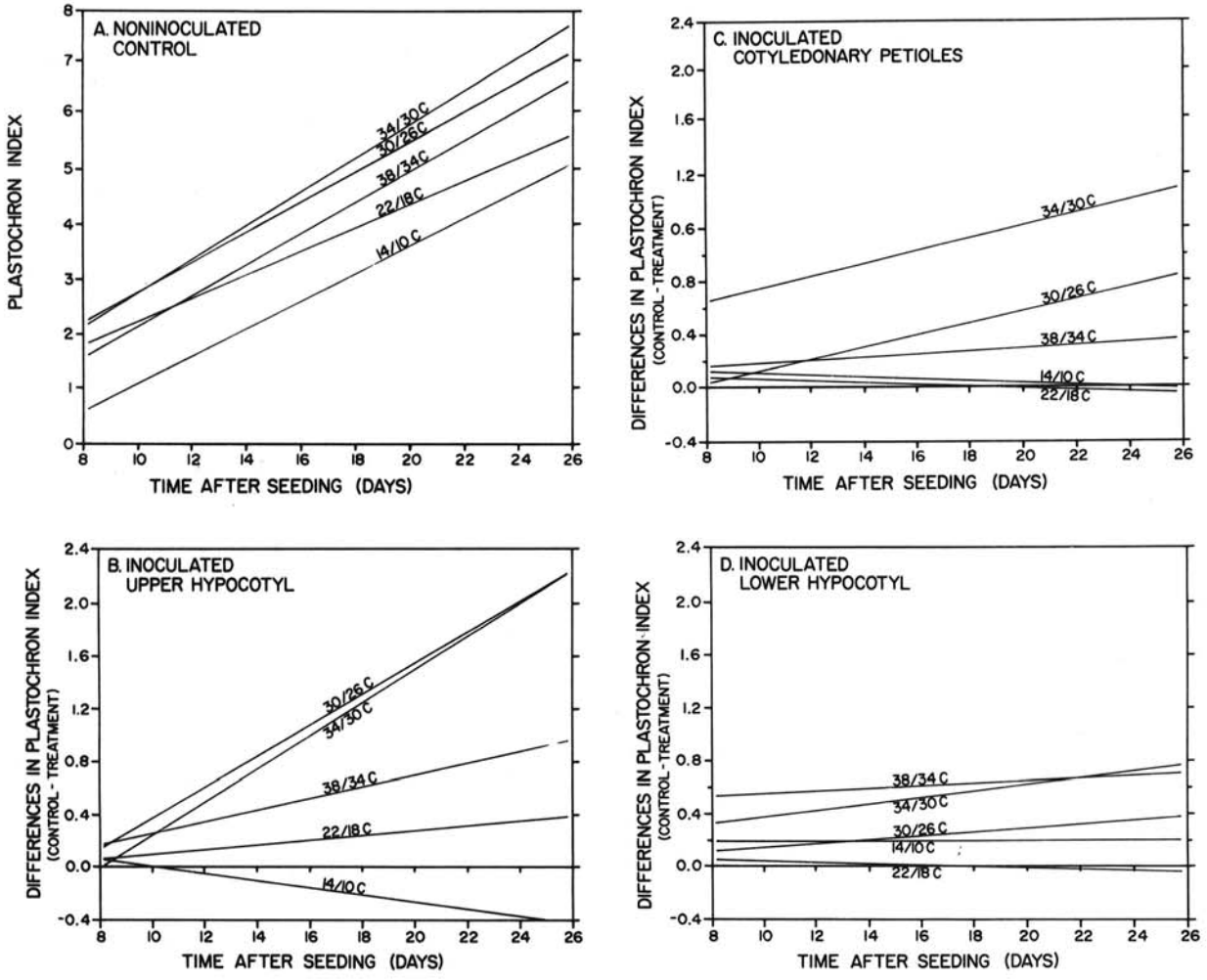


Fig. 2-(A to D). Plastochron Index (PI) measurements of plants grown at five day/night temperatures. Data were developed from six plants/treatment/temperature measured over time. The mean correlation coefficients for regression lines A, B, C, and D are 0.98, 0.90, 0.75, and 0.76, respectively. **A)** Noninfected control plants. **B), C),** and **D)** The difference in the PI of the control (A), minus the PI of the radish plants infected with *Agrobacterium tumefaciens*. A positive value indicates a decrease in the number of new leaves initiated. These data are from the same plants as those used for Fig. 1.

TABLE 1. Influence of temperature on gall formation in radish plants inoculated with *Agrobacterium tumefaciens* at three infection sites

Day/night temperature (C)	Amount of gall tissue formed following inoculation at various sites[a]					
	Cotyledonary petioles		Hypocotyl			
			Upper		Lower	
	Aboveground (%)[b]	Total (%)	Belowground (%)	Total (%)	Belowground (%)	Total (%)
14/10	2	1	0	0	0	0
22/18	16	8	44	27	9	5
30/26	42	36	48	28	41	21
34/30	42	35	42	24	37	18
38/34	13	10	60	24	82	29

[a]Gall tissue (from each of the three infection sites) is expressed as a percentage of the plant tissue where it was located and as a percentage of the total plant. ($S\bar{x}$ = 12 mg).

[b]Aboveground tissue includes leaves and shoot; belowground tissue includes the hypocotyl and roots. Mean of six plants/treatment.

temperature range. Infection in the lower hypocotyl slowed the initial weight increase, then above 30/26 C, the leaf and shoot weight decreased to 31% of the control at 34/30 C. The weight of leaves and shoots from plants infected in the cotyledonary petioles exceeded that of the control plants throughout the temperature range.

The amount of gall tissue formed at 14/10 C was negligible regardless of the infection site (Table 1), but at 38/34 C, gall tissue comprised 60 to 80% of the hypocotyl and root weight when hypocotyls were inoculated. At this temperature only 13% of the aboveground parts of petiole-inoculated plants was gall tissue. Thus, the proportion of tissue that developed into gall vs. healthy tissue is influenced by the temperature and the infection site.

Aboveground parts of plants infected in the cotyledonary petioles were visibly stunted, but their dry weights (including gall tissue) were not significantly different from those of the noninfected controls (Fig. 1-A). Galls on the petioles accounted for about 42% of the aboveground weight at 30/26 and 34/30 C (Table 1), but the total leaf areas of these plants were 32 to 35% less than the corresponding controls (Fig. 1-B). In contrast, leaf area measurements for plants inoculated through the upper and lower hypocotyl showed the same relationship to the control as did the dry weight measurements (Fig. 1-A).

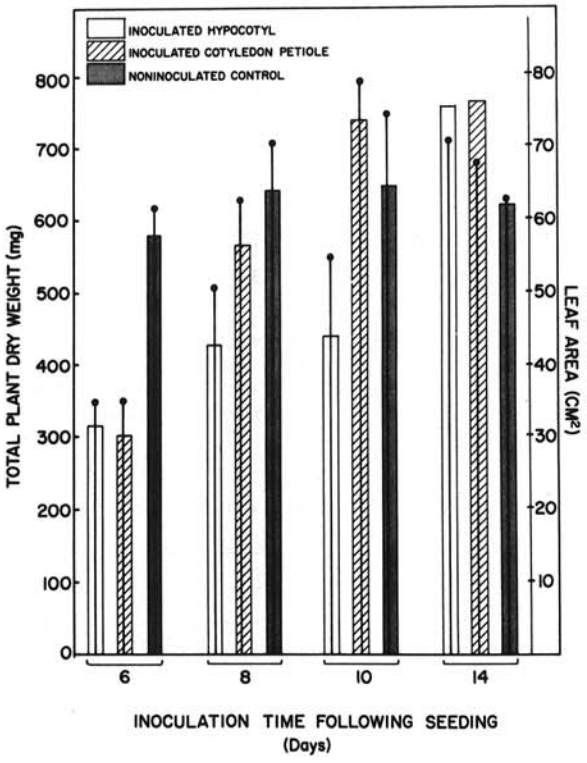


Fig. 3. Leaf area and total dry weight of radish plants inoculated at increasingly older ages with *Agrobacterium tumefaciens*. Plants were all grown at a day/night temperature of 30/26 C. Each point is the mean of six observations. Bars = dry weight ($S\bar{x}$ = 61 mg); vertical lines in the center of the bars = leaf area ($S\bar{x}$ = 6.6 cm²).

The influence of day/night temperatures on formation of belowground tissue of control plants differed significantly from that on aboveground parts (Fig. 1-C). The optimum temperature for production of aboveground parts (34/30 C) was significantly higher than that for production of roots and hypocotyl (22/18 C). This variable growth response of radish plants to temperature may account in part for the differential effect of crown gall on plant growth at different infection sites.

The weight reduction in belowground tissue of infected plants was similar for all infection sites over the entire temperature range except for the severe weight reduction of plants infected at the cotyledonary petiole and grown at 30/26 or 34/30 C (Fig. 1-C).

Influence of inoculation with Agrobacterium tumefaciens on leaf initiation.—The rate of leaf initiation in noninoculated radish plants at various temperatures was relatively constant (Fig. 2-A). A new leaf was formed every 3.3 to 4.8 days at 34/30 C and 22/18 C, respectively. Thus growth evaluated by the Plastochron Index (PI) agrees well with dry weight measurements as an estimate of aboveground growth (Fig. 1-A), and in addition provides a continuous measurement over the duration of the experiment. To our knowledge, this is the first time the PI has been used to estimate the effect of plant pathogens on plant growth. Most commonly, the PI is used to follow morphogenesis of plant organs.

With infected plants, reduction in the rates of leaf initiation was greatest with plants infected in the upper hypocotyl (Fig. 2); this effect occurred shortly after inoculation and was magnified with increasing time (Fig. 2-B). Leaf initiation in plants infected in the cotyledonary petioles (Fig. 2-C) and the lower hypocotyl (Fig. 2-D) was not decreased significantly, but the total leaf area was reduced significantly when the plants were infected in the cotyledonary petioles and the lower hypocotyl (Fig. 1-B).

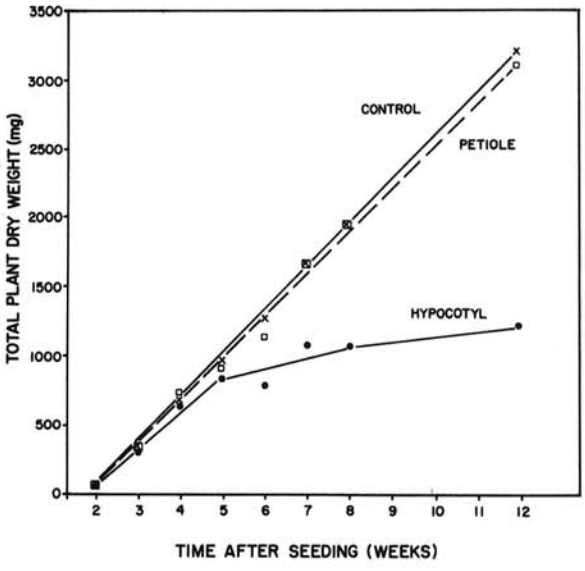


Fig. 4. Total dry weight of infected *(Agrobacterium tumefaciens)* and noninfected radish plants maintained for 12 weeks in the greenhouse at 18-25 C. Subgroups of nine plants from each treatment were harvested weekly. ($S\bar{x}$ = 80 mg).

Thus, leaf size and not leaf number was affected when plants were infected at these two sites, but both leaf number and leaf size were reduced in plants infected in the upper hypocotyl.

Influence of plant age at inoculation on subsequent growth.—Six plants grown at 30/26 C were inoculated on either the 6th, 8th, 10th, or 14th day after seeding, and growth measurements were made 22 days after inoculation. The mean dry weight of plants inoculated in the cotyledonary petioles was significantly lower than that of the corresponding controls only when inoculated the 6th and 8th days after seeding, whereas the dry weights of plants inoculated in the upper hypocotyl were significantly lower than the controls when inoculated 6, 8, and 10 days after seeding (Fig. 3). Dry weights of all plants inoculated 14 days after seeding were significantly greater than the control, even though there was no significant difference in total leaf area between treatments.

Permanency of stunting.—To determine if stunted plants could recover from infection and eventually produce the same amount of tissue as noninfected control plants, 216 radish plants were inoculated in the greenhouse 7 days after seeding. Inoculations were made in the upper hypocotyl or at both cotyledonary petioles, and nine plants were used per treatment. One-hundred and eight noninoculated control plants were included. Temperatures were uncontrolled, ranging from 18-25 C, and the plants were given no supplemental light. Plants from each of the two treatments and the control were harvested weekly to measure the total dry weight.

Although the mean total dry weight of noninoculated plants increased linearly over a 12-week period, mean total dry weight of plants that had been inoculated in the upper hypocotyl began to plateau at 5 weeks and was 62% less than the noninoculated controls at 12 weeks (Fig. 4). However, the mean dry weight of plants inoculated at the cotyledonary petiole increased linearly over time and was nearly identical to the control. If leaf area of plants infected at the cotyledonary petiole had been measured, a decrease in surface area might have been observed (cf. Fig. 1-A, B), because tumors on the petioles of these plants were relatively large. These data show that crown gall infection can cause a permanent stunting of the plant if infection occurs at the proper site.

DISCUSSION

Crown gall can seriously reduce the growth of radish plants, but the magnitude is dependent on temperature, age of the plant when infected, and the site of infection. Varying any of these factors can influence the damage from the infection and probably accounts for discrepancies in the literature relative to the effect of crown gall on plant growth and crop production. Other variables include disease resistance of different cultivars (5) and host specificity of the pathogen (11).

Of the three inoculation sites studied, infection of the upper hypocotyl usually resulted in the most severe stunting. Because infection of the hypocotyl may interfere with vascular function (6), the cotyledonary petioles also were inoculated. Any reduction in growth of plants inoculated at the petiole would probably occur because of debilitating substances produced by the tumors or preferential mobilization of materials to the tumor (4). In any event, early infection of the cotyledonary petioles of radish can result in decreased leaf area and drastically reduced belowground tissue. These data quantitatively confirm visual observations about the importance of infection sites on woody plants (5, 7).

When tumors at the cotyledonary petiole were included in the aboveground weight, there was no significant difference between infected and noninfected plants, even though leaf area of these plants was reduced about 33%. El Khalifa and Lippincott (2) observed a similar trend with inoculated leaves of beans. Length and width of infected bean leaves were 18% less than the noninfected controls, but the weight increased by 90%. They also reported that epicotyl bud growth was delayed by about 20 hours on inoculated plants, but thereafter its growth paralleled that of the controls. The final length of the bud was about 20% less than the controls. Our PI determinations showed a similar reduction of leaf development except when plants were infected in the cotyledonary petioles and in the upper hypocotyl at day/night temperatures of 30/26 and 34/30 C. In these instances, the rate of leaf initiation decreased continuously throughout the experiment.

The influence of temperature on gall formation in radish differs widely from that in tomato. Riker (8) reported that no tumors developed on inoculated tomato plants above 30 C. In contrast, galls developed on inoculated radish plants at 38/34 C, even though growth of noninfected plants was greatly reduced at those temperatures. The diurnal thermal and photoperiods used in our experiments may have allowed gall development at a higher temperature. However, Deep and Hussin (1) also reported more numerous and larger galls on inoculated roots of mazzard cherry kept at a soil temperature of 35 C for 5 days following inoculation than on similar plants kept at 25 C. Apparently, radish plants and mazzard cherry seedlings respond similarly to infection at higher temperatures.

Our work demonstrates that crown gall can seriously retard plant growth without the additional complication of heart-rotting fungi that can enter the tree via a tumor (5, 10), and provides reasons why some plants may be infected with crown gall but show no visible adverse effects. Studies are now underway to determine how growth reduction occurs, aside from physical disruption of vascular flow.

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