Latent Infections and Seasonal Variability of Crown Gall Development in Seedlings of Three Prunus species

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ABSTRACT

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Infection by *Agrobacterium tumefaciens* and subsequent symptom expression was similar on seedlings of three *Prunus* species (mahaleb and mazzard cherries and myrobalan plum) that were wounded and inoculated in the field. They were heavily infected when inoculated in June, July, and August, but earlier and later inoculations were less successful. Soil moisture had little influence on symptom expression, but low soil temperature was correlated with reduced susceptibility. Latent infections occurred the first growing season on mahaleb and mazzard cherry seedlings wounded and inoculated after September and galls developed on these plants during the second growing season. Wounds were necessary for optimum infection of all three *Prunus* species, but many nonwounded 3-year-old mazzard trees became infected when inoculated during the growing season. Wounds on stored, dormant mazzard seedlings remained susceptible to *A. tumefaciens* for 107 days, whereas less than 20% of the wounds made in late June on mazzard seedlings growing in the field became infected when inoculated 6 days later. The extended period of wound susceptibility on dormant seedlings and latent infections must be carefully considered in devising methods to prevent crown gall.

Crown gall, caused by *Agrobacterium tumefaciens*, continues to be a serious disease of nursery stock in the Pacific Northwest. In the nursery, eradicants such as Bacticin are not economical for destroying the tumors (7). Instead, control procedures must be devised to prevent infection. At present, galling is the only criterion signalling infection, and thus infection is difficult to predict in time for preventive control measures. Galls become visible macroscopically on plants in the greenhouse within 6-14 days after inoculation, but relatively little is known about infection and subsequent symptom development in *Prunus* seedlings grown in the field. Delayed symptom development during the growing season (6), and incipient infections (2), can further confuse interpretation of when infection is initiated.

The purpose of this study was to determine: (i) at what growth stage of the host galls become visible in the field, (ii) the time period between inoculation and symptom expression in the field or in winter storage, (iii) the influence of soil temperature and moisture on symptom expression, and (iv) how long wounds on *Prunus* species remain susceptible to infection during the growing season and in storage.

MATERIALS AND METHODS

A mixture of six pathogenic strains of *Agrobacterium tumefaciens* (Smith and Towns.) Conn., 1942 was routinely used as inoculum. The strains were EU-8, 3-ketolactose positive (KL^+), from M. N. Schroth, Berkeley, CA; B234, KL^-, from J. DeVay, Davis, CA; K27 and K29, KL^-, from A. Kerr, Australia; B6, KL^+, from R. Baker, Fort Collins, CO; and Q51, KL^-, isolated from a diseased cherry tree in Oregon. A preponderance of KL^- isolates was used in the inoculum because the KL^- strain predominates in the Pacific Northwest (1). None of the strains inhibited growth of the other isolates when cross-streaked on nutrient medium.

The inoculum was prepared from cultures grown in broth (YDP) containing 4 g yeast extract, 20 g dextrose, 4 g peptone, and 5 g $(NH_4)_2SO_4$ in 1 liter of distilled water adjusted to pH 7.0. Cultures in late log-phase were mixed and diluted with distilled water to 3- to 8×10^8 colony-forming units per milliliter.

Tree seedlings were planted, grown, harvested, and stored along with commercially propagated stock in nurseries at Aurora and at Sauvies Island, Oregon. In these nurseries seed was planted in October, they germinated in February or March, and seedlings were lifted in December and stored in a warehouse or "heeled-in" outside. Prior to transplanting, dormant seedlings were graded and the stem tip and the tap and lateral roots were pruned.

Field-grown seedlings of myrobalan plus, *Prunus cerasifera* (Ehrh.); mahaleb cherry, *P. mahaleb* (Linn.); and mazzard cherry, *P. avium* (Linn.) were used as host plants. For inoculations in the field, the soil was carefully removed from around the crown, and a longitudinal slit was cut 2-3 cm long on the crown with a linoleum knife. Trees in storage were wounded similarly, and in addition, the tap and lateral roots were pruned before planting. Plants were inoculated by spraying them to run-off with the inoculum suspension. Two types of controls were

used: (i) inoculated but nonwounded, and (ii) wounded but noninoculated. Fifty seedlings per treatment were used unless otherwise specified. Treated seedlings were harvested in December and the presence of crown gall was noted.

Soil moisture in the field was monitored throughout the growing season at 6, 18, and 36 cm below the soil surface using a Delmhorst Model KS meter and gypsum moisture blocks. Soil temperatures were monitored at the soil surface and at depths of 6, 25, and 46 cm using mechanical, 7-day recording thermographs and thermocouples manufactured by Freiz and Son Co., Baltimore, Maryland. The temperature and moisture sensors were placed either directly below or within 2-3 cm of the seedling tap roots.

Ten of the wounded but noninoculated control seedlings from each species were selected in April, and stem caliper and height measurements were made periodically throughout the growing season to determine if symptom development was affected by growth of the host plant.

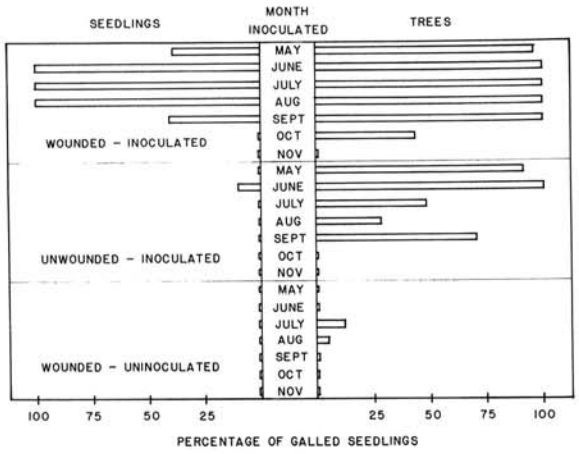

Fig. 1. Incidence of galled mazzard cherry seedlings and trees wounded and/or inoculated throughout the growing season with *Agrobacterium tumefaciens*. The seedlings were 3-10 months old grown at Sauvies Island, Oregon. The trees were grown at Aurora, Oregon, for 3 years. Data based on observations of 50 seedlings and 25 trees at each date.

RESULTS

Symptom expression during the growing season.—Symptoms were most evident at Sauvies Island when mazzard seedlings were wounded and inoculated in June, July, and August 1971 (Fig. 1). Seedlings wounded and inoculated from October to December were symptomless at harvest. Symptom development in mahaleb seedlings was similar to those in mazzard (Fig. 1), but the incidence of galls was slightly higher in the mazzard seedlings.

Symptom development was noticeably different in inoculated 3-year-old mazzard trees at Aurora. Wounding and inoculation in May, September, and October resulted in increased galling (Fig. 1). Inoculated but nonwounded trees were also relatively heavily galled. The bacteria may have entered via the numerous lenticels on the tree crowns, thus accounting for the high incidence of disease in the nonwounded trees. Wounded but noninoculated seedlings and trees were little affected by the disease.

Delayed symptom development.—Inoculated, symptomless mazzard and mahaleb seedlings were heeled-in over winter and planted in the field at Sauvies Island in May 1972, to determine if symptoms would develop subsequently. Many of these seedlings developed crown gall during 1972 (Table 1). Galls were generally present at the wounds made the previous year, but some developed where the tap and lateral roots were pruned at transplanting. Wound-healing and an early *Rhizoctonia solani* infection of the below-ground cortical tissues may have reduced wound susceptibility of the seedlings inoculated in May. Few galls occurred on the wounded but noninoculated seedlings, indicating either that natural soil populations of *A. tumefaciens* were low or that the bacteria were unable to infect the wounds.

Duration of wound susceptibility on growing and dormant seedlings.—The crowns of mazzard seedlings growing in the nursery were wounded on 28 June. Groups of 40 seedlings were inoculated at various times thereafter

TABLE 1. Crown gall development in 1972 on mazzard and mahaleb cherry seedlings that were inoculated in 1971 with *Agrobacterium tumefaciens* but were symptomless that year

1971 Inoculation dates	Percent galled seedlings—December 1972[a]					
	Mahaleb			Mazzard		
	Wounded inoculated[b]	Wounded noninoculated	Nonwounded inoculated	Wounded inoculated[b]	Wounded noninoculated	Nonwounded inoculated
6 May	18	0	0	33	2	2
5 June		0	25		0	25
6 July		0	24		0	16
7 August		0	9		0	13
6 September	80	0	11	100	0	4
6 October	93	4	0	74	0	14
8 November	80	0	0	48	0	10
5 December	50	0	0	94	15	22

[a]Seedlings were "heeled-in" outside until transplanted in May 1972. Galling was evaluated in December 1972 when the seedlings were harvested.

[b]82-100% of the seedlings inoculated in June, July, and August were galled at harvest in December 1971 and were not replanted in 1972.

over a 42-day period. At each inoculation date, control seedlings were freshly wounded and immediately inoculated or freshly wounded but not inoculated.

As expected, all growing seedlings wounded and immediately inoculated developed galls, but wound susceptibility decreased rapidly thereafter (Fig. 2). Wound callus was evident on some seedlings 6 days after wounding, by 12 days most of the seedlings were callused, and by 21 days the wounds often were completely callused over.

To determine the susceptibility of wounds on stored, dormant mazzard seedlings, the crowns were wounded on 21 December 1971 and groups of 50 seedlings were inoculated with *A. tumefaciens* at various times thereafter. The seedlings were stored in a nonrefrigerated warehouse until April 1972 when they were planted in the field. In December 1972 the seedlings were harvested and rated for the incidence of galls.

The wounds of over 60% of the seedlings remained susceptible to *A. tumefaciens* for 107 days, but by 113 days there was little galling (Fig. 2). In the controls, 84% of the seedlings wounded and inoculated on the day of planting developed galls, but only 7% of the wounded noninoculated seedlings became galled.

In a subsequent experiment, pruned roots of mazzard seedlings were heated in moist sawdust for 3 weeks at 17-20 C to accelerate wound callusing. The stem and buds were kept at 1-3 C to prevent bud break. Only 6% of these heat-treated seedlings were naturally infected at harvest compared to 64% of the nonheated seedlings.

Symptom expression as related to soil moisture and temperature and seedling growth.—The seasonal pattern of crown gall development in *Prunus* seedlings inoculated from April through December of 1972 (Fig. 3-A) was similar to that for mazzard seedlings inoculated in 1971 (Fig. 1). The number of galled seedlings increased rapidly in May, remained high for about 3 months, then declined rapidly. The rapid increase and decline of galling followed an increase and decrease, respectively, of soil temperatures at 5-7 cm below the surface (Table 2).

A factor that may have contributed to a lower incidence of galling in late April and early May was the development of elongated, reddish-brown, dry cortical lesions over the crown of most of the seedlings. Similar lesions were observed in May 1971. About mid-May the cortical lesions usually were sloughed off when seedling growth became more vigorous as soil temperatures increased. Isolations from lesions on 16 of 20 randomly selected seedlings yielded cultures of *Rhizoctonia solani*.

Soil moisture tension 5-7 cm below the surface ranged from 0.2 to 15 bars and had little relation to symptom expression. Apparently, galls can develop on the crown at

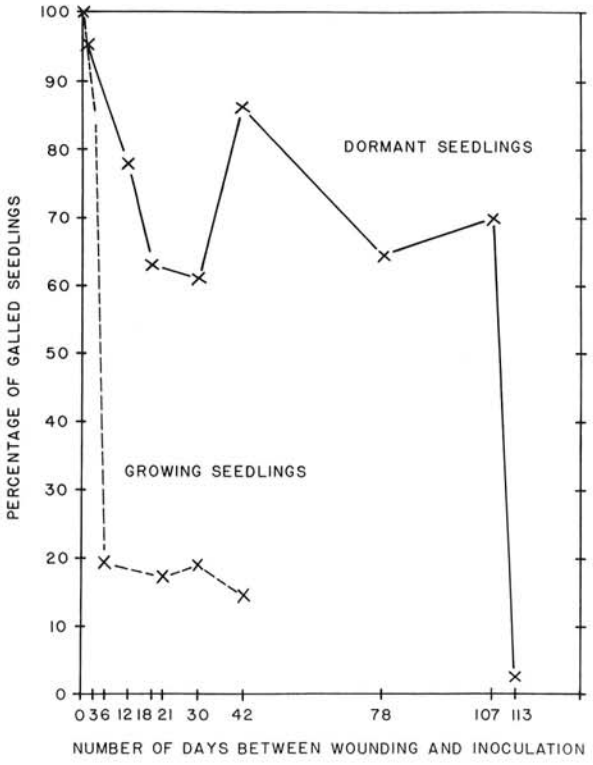

Fig. 2. Duration of susceptibility of wounds to *Agrobacterium tumefaciens* on dormant and growing mazzard cherry seedlings. All seedlings were wounded on the crown at zero time and inoculated periodically thereafter. Data based on observations of 50 dormant seedlings and 40 growing seedlings inoculated at each date.

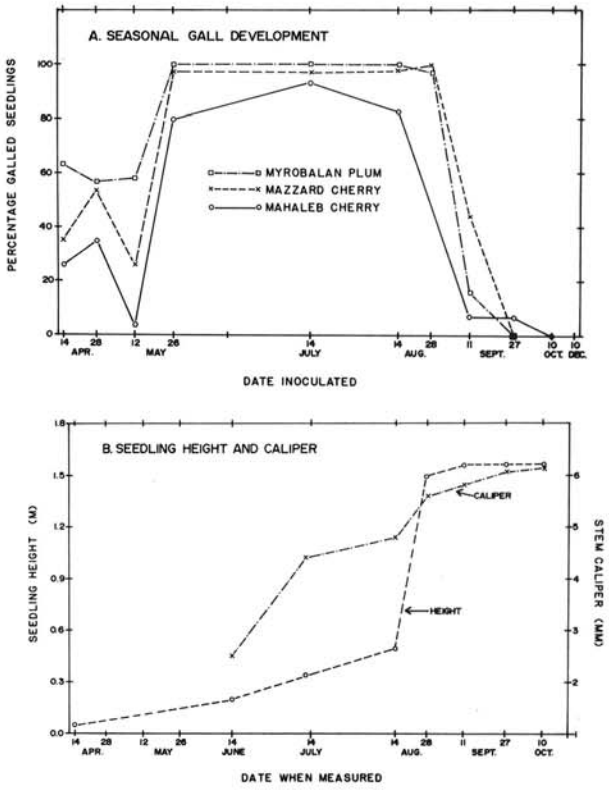

Fig. 3-(A, B). A) Crown gall development on three *Prunus* species wounded and inoculated with *Agrobacterium tumefaciens* during the growing season. **B)** Stem caliper and height of 10 wounded, noninoculated mazzard cherry seedlings that were selected in April and measured periodically thereafter. Growth curves of myrobalan plum and mahaleb cherry were nearly identical to that of mazzard cherry.

low soil moisture tensions as long as the seedlings continue to grow.

No relation was detectable between symptom expression and seedling growth. Mean increases in seedling height and stem diameter were similar for all three species (Fig. 3-B). The decrease in gall incidence in late August occurred during the onset of bud dormancy, but the stems continued to increase in diameter until 15 November (Fig. 3-B), indicating that the lateral cambium was active and potentially capable of developing galls even though none was visible after 27 September.

DISCUSSION

Oregon nurserymen occasionally find crown gall on *Prunus* seedlings during the first growing season, and the incidence increases sharply after transplanting the next year. The question then is whether young host tissue is less susceptible, or does handling and wounding during transplanting favor increased natural infections?

This study shows that young *Prunus* seedlings wounded and inoculated with *A. tumefaciens* are highly susceptible the first growing season. In contrast, natural infections occur regularly on mazzard cherries, but only rarely on mahaleb in Oregon. Although wounds on young seedlings of all three species are equally susceptible to *A. tumefaciens,* there are as yet unidentified factors that preferentially favor natural infection of mazzard cherries.

The seasonal symptom pattern of *Prunus* seedlings following inoculation with *A. tumefaciens* was similar to that reported for crown gall and hairy root on young apple trees (3, 6), even though susceptibility was judged differently with apples. The higher incidence of galled *Prunus* seedlings and shorter incubation period for apple tree infection both occurred as soil temperature increased. No symptoms developed as soil temperatures decreased and dormancy began.

Riker and Hildebrand (6) and Hildebrand (3) thought the onset of dormancy stopped crown gall and hairy root development on apple trees, and in *Prunus* seedlings apical bud dormancy was coincident with reduced susceptibility. However, when dormant *Prunus* seedlings were wounded and inoculated with *A. tumefaciens* in the field in October and November and immediately transplanted and grown in a greenhouse at 21-24 C, galls developed in 8-12 days, but none developed on similar plants kept at 10 C (L. Moore, *unpublished*). Thus, low temperatures and not dormancy appear to retard gall initiation.

Deep and Young (2) concluded that latent infections occurred on pruned roots of mazzard seedlings that had been dipped for 3 minutes in a solution of 1.05% sodium hypochlorite and planted in autoclaved soil. However, the pathogen may have been on the root (5) unaffected by the sodium hypochlorite, and then infected the pruning wounds. I have observed increased galling when wounded and inoculated roots of mazzard seedlings were dipped in a solution of 1.05% sodium hypochlorite for 15 minutes (L. Moore, *unpublished*).

Hildebrand (3) reported that wounded underground stems of apple trees inoculated with *A. rhizogenes* in mid-September remained symptomless until May when all the trees developed hairy root symptoms. He speculated that the pathogen overwintered in the wounds. Some *Prunus* seedlings that were wounded and inoculated in May and were symptomless in December, developed galls the following growing season on the wounded crowns even though the wound-site was callused over. These late-developing galls cannot be attributed solely to infection by resident *A. tumefaciens* because galling was negligible or absent on the inoculated but nonwounded control seedlings. Possibly root pruning and the physiological stress of storage and transplanting helps activate the latent infections.

This study demonstrates that *Prunus* spp. roots can become infected anytime during the nursery operation, but symptoms will only be expressed under particular conditions. Soil temperature is the most critical environmental factor effecting gall development on roots of growing seedlings.

Carelessness in harvesting and handling seedlings can provide wounds that remain susceptible to *A. tumefaciens* residing on the roots (5) or coming from some contaminating source (8). These latent infections respond poorly to treatments with bactericides, particularly if tumor transformation already has been initiated. Furthermore, preplanting dips to protect pruned roots against natural inoculum in cool soils will require chemicals with a long residual toxicity to bacteria because of slow wound healing. Effective protection may be achieved by manipulation of the resident *Agrobacterium* population of the roots in favor of an antagonistic, nonpathogenic *A. radiobacter* following inoculation of the seed (4).

TABLE 2. Soil temperatures for the early and latter parts of the 1972 growing season

Mean weekly soil temperatures[a]			
Spring		Autumn	
Date	Temp	Date	Temp
15-22 April	8	7-14 September	18
23-30 April	10	15-22 September	14
1-8 May	13	23-30 September	12
9-16 May	16	1-8 October	11
17-24 May	16	9-16 October	11

[a]Data were calculated by summing the daily maximum and minimum temperatures and dividing by two. The temperature sensors were positioned 5-7 cm below the soil surface and 2-3 cm from the seedling tap root.

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