Influence of Inoculum Dosage, Time After Wounding, and Season on Infection of Persian Walnut Trees by Erwinia rubrifaciens

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This work was supported by ARS, USDA Grant No. 12-14-100-9930(34) to C. I. Kado.

The authors thank George C. Martin, Department of Pomology, University of California, Davis, for his cooperation and contribution of the walnut orchard. Technical assistance of John Uyeyama and Glenn Kurimoto is gratefully acknowledged.

Accepted for publication 19 September 1972.

ABSTRACT

Experimentally inflicted wounds of Persian walnuts (*Juglans regia* 'Hartley') are susceptible to infection by *Erwinia rubrifaciens*. Susceptibility depends on season of the year; time of adding inoculum to the open wound; and the amount of the inoculum. Hartley trees were not susceptible during the winter (January), but were highly susceptible in spring (April), summer (July), and fall (October) months. No infection occurred in January even at high levels of inoculum (graded inoculum doses up to 10^6 cells/ml were used). Very few *E. rubrifaciens* cells (5-10 cells/wound) were needed to establish infection in April, July, and October. Wounds decreased in susceptibility with time at the average rate of 10-12%/day. Seven days after wounding, fewer than 3% of wounds remained susceptible in April and October, and about 15% in July. The implications of these results are discussed.

Phytopathology 63:327-329

Erwinia rubrifaciens Wilson, Zeitoun, & Fredrickson infects the highly susceptible Hartley cultivar of Persian (English) walnut tree (*Juglans regia* L.), causing "deep bark canker". The bacterium primarily invades nonfunctional secondary phloem tissues (2), and spreads in the phloem causing extensive necrosis of the inner bark of the trunk and main branches (4). The present paper reports the susceptibility of wounds as a function of time of year, time elapsed between wounding and inoculation, and inoculum dosage.

MATERIALS AND METHODS.–*Preparation of the inoculum.*–Cells of *E. rubrifaciens* 6D34 were grown in shake culture at 30 C in medium 523 (1). The cells were collected during exponential growth, washed with sterile water, and adjusted to approximately 2×10^8 cells/ml. The number of viable cells in each inoculum was determined by plating dilutions on yeast-glucose-calcium-carbonate agar plates. The freshly prepared inoculum was kept chilled in crushed ice and used within 6 hr.

Test host.–Ten- to 12-year-old Hartley trees located in a disease-free orchard on the Armstrong Ranch, University of California, Davis, were used. The trees were on rootstocks of either black walnut (*J. hindsii* Jeps.) or Paradox hybrid (*J. hindsii* × *J. regia*).

Inoculation procedure.–Deep vertical wounds (2-3 inches long) were made with a wood chisel into the inner phloem. Ten-15 wounds, made 1-3 ft above the graft union, were spaced 3-4 inches apart around the trunk of each tree. Inoculum (50 μliters) was placed into each wound with a Hamilton syringe (Hamilton Co., Whittier, Calif.). For time-course inoculation experiments, all wounds were made on day 1 and inoculum was added at time zero and at various time intervals (days) thereafter. Trees for the inoculum-dosage experiments were wounded and immediately inoculated with varying concentrations of inoculum. All inoculations were replicated at least 8 times on separate trees. A total of 68 trees was used.

Verification of infection.–Trees were examined for infection in August of the following year by the stripping away of bark with a draw knife. Samples of tissue from each wound site were placed in sterile water to allow exudation of bacteria. One-tenth-ml samples were spread on yeast-glucose-calcium-carbonate agar plates containing cycloheximide (100 μg/ml). The plates were incubated at 30 C and the pathogen was detected by the characteristic water-soluble red pigment diffusing from bacterial colonies.

RESULTS.–*Effect of time of year.*–If susceptibility of Hartley trees to *E. rubrifaciens* is less at certain times of the year, it would be advantageous to do pruning work and other cultural practices that produce open wounds on limbs and trunk when tree susceptibility is least. Also, appropriate contraindications can be recommended for the time of year when trees are highly susceptible. Hartley trees were inoculated in July and October 1970 and in January and April 1971, with suspensions containing about 10^5 *E. rubrifaciens* cells/ml. The inoculated sites were examined on 29 September 1971. A second set of inoculations were made in January 1972 and checked in June 1972. Figure 1 shows that Hartley trees were susceptible (38 to 46%) during the months of July, October, and April, but were virtually immune to infection during January (no infection from 164 inoculations).

Inoculum dosage as a function of time of year.–We have observed in field surveys that various types of bacterial cells including the pathogen occur in variable concentration in sap exuding from cracks in the bark adjacent to cankers. However, very few or no cells of the pathogenic bacterium can be recovered from the surface of bark from uninfected regions of diseased trees. Thus, if the organism is spread by

PHYTOPATHOLOGY for February (63:199-326) was issued 22 February 1973

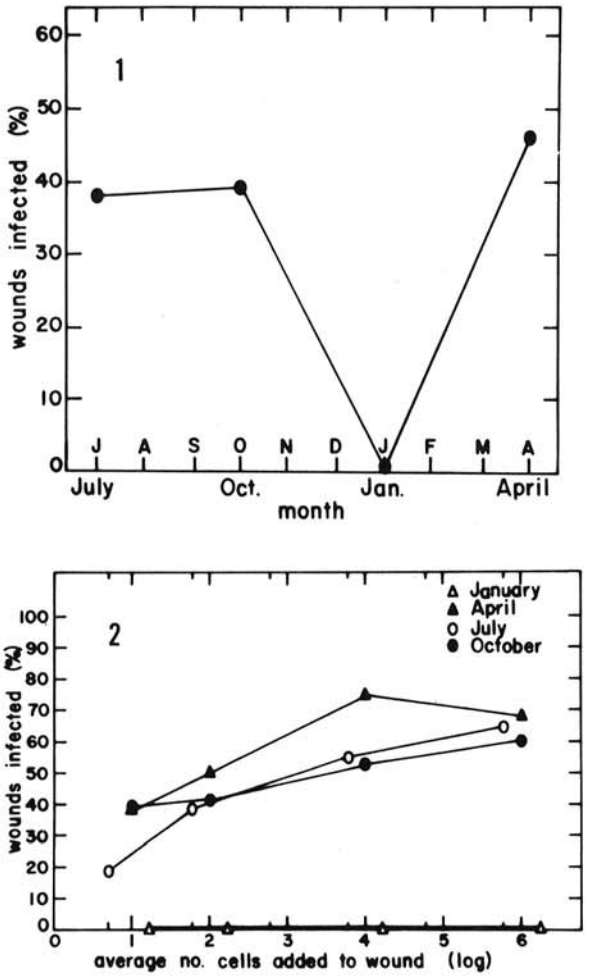

Fig. 1-2. 1) Percent wound infection of Hartley walnut trees by *Erwinia rubrifaciens* as a function of season of year. A total of 32 replicated wounds was made for each season on 16 separate trees. Inoculum (50 μliters of 10^6 cells/ml) was added to each wound. Percent expressed as number of wounds infected per total number of wounds made × 10^2. 2) Percent wound infection at various times of the year as a function of the number of *E. rubrifaciens* cells added per wound. A total of 32 replicated wounds was made for each graded dose of inoculum on 20 separate Hartley trees. Percent expressed as in Fig. 1.

splashing rain water as postulated by Schaad & Wilson (3), healthy trees distant from a diseased tree probably would receive few bacteria. To determine whether low numbers of *E. rubrifaciens* cells can elicit infection, the following experiment was done. Graded doses of inoculum ranging from 10^1 to 10^6 cells/ml were used for inoculation of various wound sites on 34 trees. Inoculations were made on 6 July 1970, 16 October 1970, 5 January 1971, and 26 April 1971, to determine differences in susceptibility due to seasonal influences (Fig. 1). The inoculated sites were examined for infection on 28 and 29 September 1971. A second set of inoculations was made on 25 January 1972. This set was examined for infection on 14 June 1972. The data in Fig. 2 show that less than 10 viable cells of *E. rubrifaciens* resulted in infection of 20-40% of the wound sites when inoculations were made in April, July, and October, but no infection resulted from inoculations made in January even when much higher doses of inoculum (10^6 cells/ml) were used. This was confirmed by repeated experiments in January 1972.

Susceptibility of wounds as a function of time.—Relatively deep wounds exposing the inner phloem tissue are required for infection (2, 3, 4). Circumstantial evidence and direct observations indicate that mechanical shakers often inflict deep wounds and serve as an effective disseminator of the disease organism (J. M. Gardner & C. I. Kado, *unpublished data*). Whether such wounds are susceptible to immediate infection or long-term (weeks) infection was examined experimentally. Figure 3 shows that wounds made during October and April were not very susceptible to infection after 7 days (less than 3%), but wounds made in July remained farily susceptible (15%) for at least 7 days. As in previous experiments, there was no infection of wounds made in January regardless of when or how

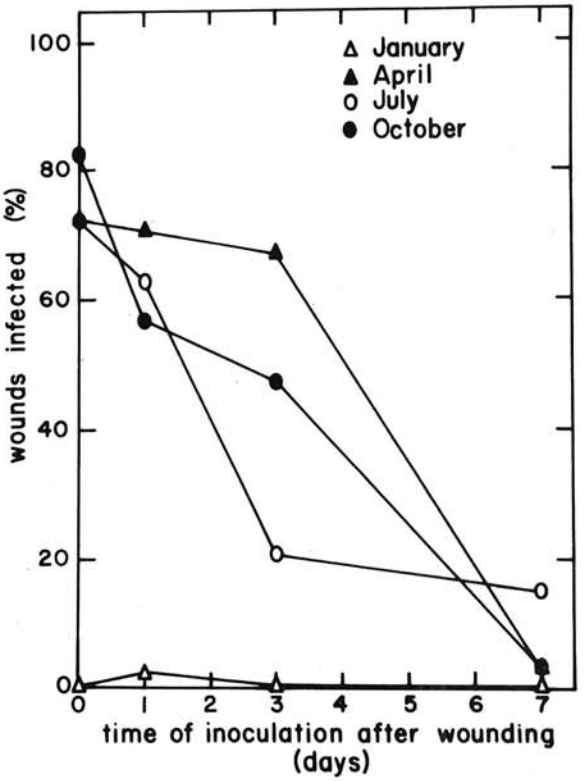

Fig. 3. Wound susceptibility of Hartley walnut trees to *Erwinia rubrifaciens* as a function of time of inoculation after wounding and season. Twenty-seven replicated wounds were made for each season (a total of 108 wounds) on 20 separate trees. Inoculum (50 μliters of 10^6 cells/ml) was added on the days indicated. Percent expressed as in Fig. 1.

much inoculum was added. The average rate of decreasing susceptibility of wounds was about 10-12%/day, as calculated from pooled data of April, July, and October.

DISCUSSION.—These results show that experimentally made wounds are highly susceptible to infection by *E. rubrifaciens* during April, July, and October, but not in January. The susceptibility of freshly made wounds in October is important because this is harvest time and we have observed that many infection sites are associated with wounds created by mechanical harvesters (shakers). This is supported by evidence showing transmission of bacteria with the shaker (J. M. Gardner & C. I. Kado, *unpublished data*). The possibility still may exist, however, that wounds made by other agents (e.g., bark damage caused by sap-sucking birds, scales, and wounds made by pruning) can serve as infection sites. Since primary sites of infection are more commonly associated with shaker wounding than with other types of wounding, the shaker appears to be the primary suspect responsible for wounding and inoculation transmission. The possible role of splashing rain still remains because the present results show that few bacteria (5-10 cells/wound) can elicit infection (Fig. 2), and earlier studies showed that 200-300 bacteria can be spread 24-26 ft from infected trees by splashing rain (3). However, the likelihood of dissemination of the disease by rain seems remote because early fall rains are not common in the central valley of California. Rains that occasionally occur during October would have to coincide with the brief wound susceptibility period. Our data show that wounds decrease in susceptibility at the average rate of 10-12%/day during October, remaining susceptible only about 7 days, and not all deep wounds are infectible during this month. Spread could occur by late-season rains, but disease spread is most likely a result of the mechanical shaker.

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