Budwood Transmission of *Erwinia rubrifaciens*, Causal Agent of Deep Bark Canker Disease of English Walnut

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ABSTRACT

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Deep bark canker disease of English walnut (*Juglans regia*), caused by *Erwinia rubrifaciens*, was observed on 181 of 1,640 cv. Hartley trees in an orchard the first summer after planting. On most infected trees, external symptoms of disease were masked by subsequent growth and were not observed again until 5 yr later when 27 trees had symptoms. Of these 27 trees, 19 had symptoms in the first year. Three of 102 and one of 120 trees that grew from budwood collected from mature diseased trees and grafted to Northern California black walnut (*J. hindsii*) rootstock developed internal and external symptoms, respectively. In two experiments, the pathogen was recovered from four of 120 and 14 of 100 asymptomatic trees. Symptoms were not observed on trees grafted with budwood from healthy trees. Healthy budwood dipped or soaked in cell suspensions of the pathogen or cut with a contaminated knife was rejected when grafted, whereas noninoculated control grafts were successful.

Deep bark canker is a bacterial disease of English walnut trees (*Juglans regia* L.) caused by *Erwinia rubrifaciens* Wilson et al (18). Most walnut cultivars are susceptible, but the widely planted Hartley is most commonly afflicted (7). Diseased trees develop short, longitudinal cracks in the bark of the trunk and main scaffolds from which a watery, reddish brown exudate flows, especially during summer and fall. The disease is chronic, and as infection advances slowly up the tree, cracks become dry and no longer produce ooze. In time, affected limbs lose vigor, foliage is reduced, leaves senesce early, and in severe cases, the limbs die. However, deep bark canker usually is not a primary cause of tree death.

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E. rubrifaciens resides in the secondary nonfunctional phloem and ray parenchyma (14). A section through the phloem and outer wood, tangential to the long axis of the tree, reveals black streaks in the inner phloem and small necrotic "pits" in the wood. The pits are infected rays seen in cross section. The pathogen is readily isolated from these tissues.

Bacteria must be deposited at the inner bark to establish infection (15). Unbroken tissue, natural openings, or shallow wounds are not suitable portals of entry. Deep wounds caused by mechanical harvesting equipment and sap-sucking birds can be infection sites, and transmission of *E. rubrifaciens* by harvesting equipment has been demonstrated (8). However, deep bark canker also occurs on trees that have never been mechanically harvested or that bear no obvious injuries that meet the requirements of an infection court.

The influence of stress in disease development is not clearly understood. In many orchards in California, trees under water stress seem more subject to deep bark canker than healthy, vigorous trees. In some cases, disease severity is mitigated during moderate summers or when growers alter cultural practices to enhance tree vigor. Occasionally, all evidence of active disease (i.e., exudation from cankers) ceases, but *E. rubrifaciens* can be cultured from necrotic pits in wood. In Tulare County, CA, irrigation practices that alleviated water stress of walnut trees over a 3-yr period reduced disease severity, as measured by the number of bleeding cankers per tree (17). Similar beneficial effects of adequate irrigation on control of bacterial blight

of filbert trees caused by *Xanthomonas corylina* (Miller et al) Dye in Oregon were reported by Moore et al (12).

Deep bark canker is usually found on walnut trees more than 8 yr old. Younger trees may escape disease because young tissue resists infection by *E. rubrifaciens* (15) and nonbearing trees are not subject to bark injuries by the mechanical harvester or to competitive stresses common among large and crowded trees. In 1976, we discovered deep bark canker on Hartley trees (with trunk diameters approximately 2.5–4.0 cm) in their first summer after planting in an orchard in Sutter County, CA. This unusual occurrence was attributed to the following probable stresses: 1) failure to top the transplants when planted, which could lead to an imbalance in the root:shoot ratio and discourage vigorous growth of new shoots, 2) abundant growth of weeds at the bases of trees, and 3) high salinity of irrigation water. The symptoms of deep bark canker disease occurred near the graft union of these young trees. There were no obvious injuries associated with the lesions, and graft transmission of the pathogen was considered. Transmission of *E. rubrifaciens* during grafting could result from an accidental inoculation of the graft cut by bacteria on a contaminated grafting knife or by resident populations in the budwood. In this paper, we describe attempts to transmit the pathogen by grafting healthy rootstock trees with naturally and artificially contaminated budwood.

MATERIALS AND METHODS

Observations of orchard. Deep bark canker symptoms were observed in 1976 in a commercial walnut orchard of 1,640 first-leaf Hartley trees on Northern California black walnut (*J. hindsii* (Jeps.) Jeps. ex R. E. Smith) rootstock in Sutter County, CA. Trees with external symptoms were mapped in the summers 1976–1982, 1985, and 1989. Diseased tissues with necrotic phloem and pits located near the graft union were collected for isolation of the pathogen in 1976 from five trees with external symptoms of disease. In 1977 and 1979, three trees that apparently had recovered from the disease were sampled.

Budwood and grafting. One-year-old wood was collected in January from 14-yr-old Hartley trees in a commercial orchard in Tulare County and stored in damp sawdust at 1 C until use in the spring. At that time, the wood was cut with hand shears, which were rinsed in alcohol and flamed between cuts, into 10- to 15-cm sections, each bearing two or three buds (budwood). A diagonal cut (graft cut) was made across the basal end of each section, a similar cut was made across the apical end of the rootstock, and the two cut surfaces were joined and secured with tape and Treeseal (Morrison Orchard Supply, Yuba City, CA). The grafting knife was variously surface-sterilized and contaminated between cuts as described below. Budwood from trees showing symptoms of deep bark canker (diseased budwood) and that from symptomless, healthy trees (healthy budwood) was grafted to black rootstock in the nursery or field without surface sterilizing the budwood.

Inoculum. A fresh culture of *E. rubrifaciens* was isolated each year from a diseased tree. The bacterium was identified by color change of Miller-Schroth (MS) medium and production of red pigment on yeast-dextrose-calcium carbonate (YDC) media (11,18). Cultures were maintained on the latter medium at room temperature (20–22 C). For preparation of inoculum, bacteria from 2- to 4-day-old cultures were suspended in sterile deionized water. Concentrations were adjusted by dilution and determined by plate counts.

Pathogenicity tests. Approximately 0.1 ml of an aqueous suspension of bacteria, 10^6 cfu/ml, was injected in small cuts into the inner bark of 16- to 19-yr-old mature Hartley trees grown at the Kearney Agricultural Center, Parlier, CA. Inoculations were made during spring or summer and trees were examined for internal symptoms in the fall.

Isolation of *E. rubrifaciens*. Sections of budwood or scions were submerged in a 1:10 solution of 5.25% sodium hypochlorite for 15–30 min, the excess moisture was allowed to air-dry for about 5 min, and the outer bark was removed with a sterilized potato peeler. A band of the entire circumference of inner bark, 2–3 cm wide, was removed and soaked for 30 min in 3 ml of sterile deionized water. The tissue was triturated with a mortar and pestle, and three transfer loopfuls of the liquid were streaked onto each of two culture plates of YDC and MS media and incubated for 7 days at room temperature.

Transmission by naturally contaminated budwood. Six rows of 60 rootstock seedlings were planted at the University of California Kearney Agricultural Center in January 1977. The rows and trees were 11 and 1 m apart, respectively. Rows were divided into five 12-tree plots to provide five replications of six treatments in a randomized complete block design. The treatments were stressed or nonstressed trees graft-inoculated by one of three methods, as follows. In March 1977, the trees were grafted with the following: 1) diseased budwood cut with a knife dipped into sterile deionized water immediately before the graft cut was made (clean knife), 2) healthy budwood cut with a knife previously dipped into an aqueous cell suspension of *E. rubrifaciens* (10^6 cfu/ml), and 3) healthy budwood cut with a clean knife. Unsuccessful grafts were repeated within 2 mo after the first attempt. One year later (April 1978), trees in each stressed treatment were undercut with a disk and the first year's growth was not topped. All other farming practices were standard and uniform for all treatments. Trees were observed for symptoms of deep bark canker at bimonthly intervals until November 1978. At that time, the outer bark was removed from 10 cm below and 30 cm above the graft union of all trees. The portion above the graft union included the budwood and the lower part of the resultant scion shoot. Tissues with suspect internal symptoms were tested for *E. rubrifaciens*.

One hundred rootstock seedlings were grafted with diseased budwood in the spring of 1980 in a commercial nursery in Tulare County, CA. In December 1980, when commercial trees would be dug for sale, the original budwood and the adjacent 15 cm of the new shoot were sampled and tested for the pathogen. On some trees, growth of the new shoot obscured the original budwood. Thus, tissues from the new shoot only (28 trees), the juncture of budwood and new shoot (25 trees), or original budwood and new shoot separately (47 trees) were tested for the pathogen. Three presumptive cultures from the experiment were tested for pathogenicity.

In winter 1981, 120 rootstock seedlings were commercially grafted with diseased budwood in the nursery, and in January 1982, the trees were planted in the field at the University of California Lindcove Field Station in Tulare County. The trees were drip-irrigated until well established in early summer, then water was withheld until leaves began to wilt. Normal irrigation was resumed and the trees recovered. In December 1982, the original budwood plus the first 15 cm of the new shoot were collected from each tree, examined for symptoms, and tested for *E. rubrifaciens*.

Effect of contamination of budwood and graft knife on transmission of *E. rubrifaciens*. Budwood was inoculated either by infestation of the graft cut or the use of a contaminated grafting knife. Budwood was dipped in an aqueous cell suspension (10^6 cfu/ml) of *E. rubrifaciens* immediately after the graft cut was made (inoculum dip) or the knife was dipped into the suspension just before the cut was made (contaminated knife). In the control treatments, the knives were dipped into sterile deionized water (clean knife) or not dipped (dry knife). Between each cut, the knife was rinsed in alcohol and flamed.

Internal inoculation of budwood. The basal ends of budwood were rinsed once or soaked for 24 hr at room temperature in water alone or in suspensions of 10^3 or 10^6 cfu/ml of *E. rubrifaciens*. Additional budwood was left untreated as a control. Six budwood pieces that had soaked for 24 hr in the bacterial suspensions were tested for presence of the pathogen. Six pieces of budwood of each treatment were wrapped in damp paper towels and incubated at 1 C for 24 and 72 hr. After each storage period, a 2-

cm-wide band of inner bark, 1 cm above the dipped or soaked end of each section of budwood, was removed and tested for the pathogen. A 1-cm-square patch of external bark surface was removed from each piece before and after surface sterilization, agitated in 3 ml of sterile deionized water with a vortex, and the liquid was spread on plates as described earlier.

Transmission by inoculated budwood. Rootstock trees, planted in January, were grafted in March 1978 with healthy budwood that had been infested with 10^3 or 10^8 cfu/ml of *E. rubrifaciens* by the inoculum dip or contaminated knife methods. Control trees were grafted with healthy budwood cut with a clean or dry knife or the graft cut of healthy budwood dipped into sterile deionized water. There were 10 single-tree replications of each treatment in a randomized complete block design. Graft survival was determined in June by the presence of a shoot.

Sixty rootstock trees grown at the Lindcove Field Station were grafted with healthy budwood soaked for 24 hr in 10^6 cfu/ml of *E. rubrifaciens*. Grafting began at the end of the 24-hr soak period and was completed within 6 hr. Rootstock trees grafted with untreated healthy budwood served as controls. Trees were observed for graft success or symptoms of disease; tissues were cultured to detect the pathogen.

RESULTS

Observation of the orchard. The incidence of deep bark canker found in the commercial orchard the first summer after the trees had been planted was 11%. Four diseased trees died during 1977, leaving 177 for the remainder of the observation period. In 1977, symptoms were observed on only eight trees, whereas none were seen from 1978 through 1980. However, in 1981, 27 trees had symptoms. Of these 27, eight were free of symptoms in 1976. In 1989, symptoms were present on 69% of the trees found diseased in 1976. In contrast, only 37% of the trees in the entire orchard had symptoms in 1989. *E. rubrifaciens* was isolated from all trees sampled each time (five in 1976, three each 1977 and 1979).

Transmission by naturally contaminated budwood. Scattered trees in all treatments died of undetermined causes, perhaps transplant shock, during the first growing season. Ninety-eight and 99%, respectively, of surviving trees grafted with healthy or diseased budwood cut with a clean knife formed successful unions, but all original grafts and regrafts made with an infested knife aborted. At the end of the experiment, 102 trees derived from grafts made with diseased budwood and a clean knife remained. Of these, necrotic pitting was found in wood of one stressed and two nonstressed trees. Pathogenic strains of *E. rubrifaciens* were recovered from these three trees. None of the trees had external symptoms; trees from grafts with healthy budwood also did not have internal symptoms.

External and internal symptoms did not develop on any of the trees grafted with diseased budwood in the spring and grown in the nursery until December 1980. However, *E. rubrifaciens* was isolated from 14 of the 100 trees tested. The pathogen was recovered from three of 28 trees from which only new shoots were sampled. Among the 47 trees having budwood and new shoot tissues cultured separately, the pathogen was recovered from both budwood and shoot of two, budwood only of four, and new shoot only of one. Four of 25 trees sampled at the juncture of budwood and the new shoot also tested positive for *E. rubrifaciens*. Trees inoculated with the three strains tested for pathogenicity produced internal symptoms typical of deep bark canker. Only one of 120 trees grafted with diseased budwood and grown for one season at Lindcove Field Station developed slight external and extensive internal symptoms of deep bark canker. *E. rubrifaciens* was isolated from this and four other symptomless trees.

Transmission by inoculated budwood. All grafts with budwood inoculated by inoculum dip or a contaminated knife aborted, whereas successful unions were formed in all control treatments. *E. rubrifaciens* was recovered immediately after treatment from half or fewer and from all budwood pieces soaked for 24 or 72 hr in suspensions of 10^3 and 10^6 cfu/ml, respectively. However, the pathogen could not be detected in surface-sterilized budwood or the water-soaked or untreated controls. Budwood soaked for 24 hr in 10^6 cfu/ml of *E. rubrifaciens* was rejected when grafted. At these graft unions, a reddish exudate appeared, followed by desiccation and death of the budwood. The exudate did not appear in other experiments when the contaminated knife or inoculum dip techniques were used. We randomly selected 12 rejected budwood pieces and tested them for the pathogen. The tissues were dry and *E. rubrifaciens* was not recovered.

DISCUSSION

E. rubrifaciens was transmitted by budwood. However, in two experiments with diseased budwood, only a low percentage of trees developed internal or external symptoms. Based on symptom expression, the level of transmission documented was low. But in commercial orchards, external or internal symptoms seldom are observed on young trees and have never been seen on 1- to 3-yr-old wood of mature trees. Inoculation by injection of inner bark of 1-yr-old wood does not result in infection (15). Furthermore, the influence of environmental factors on disease expression would affect the number of trees with symptoms.

Somewhat greater levels of transmission were found in symptomless individuals. Other symptomless carriers perhaps could have been detected by techniques more sensitive than those we employed. Extremely low populations of bacteria have been implicated in systemic spread of other pathogens (3,6,16) and a similar situation may apply here. Amounts of transmission also would be affected by uneven distribution of bacteria in the source tree and the budwood procured from it.

All attempts to transmit the pathogen with inoculated budwood failed. We demonstrated that *E. rubrifaciens* can move into inner bark tissues through the basal end of budwood pieces, but budwood inoculated in this way was rejected by the rootstock when grafted. Concentrations of bacteria lower than those we tested may prove able to colonize budwood through the graft cuts without interfering with formation of a graft union.

Natural internal contamination of budwood would require movement of bacteria into symptomless tissue. Latent, systemic movement of pathogenic bacteria in plants is well recognized (1,2, 5,9,10,13). Gardner and Kado (4) detected *E. rubrifaciens* in walnut phloem 300 cm beyond symptomatic tissue collected from trees inoculated in June or August. Trees inoculated in January or February did not develop symptoms, but 6 mo later, bacteria were recovered 105 cm from the inoculation site. Thus, systemic spread occurred in both symptomatic and asymptomatic trees.

The hypothesis that *E. rubrifaciens* is transmitted by resident internal latent populations in budwood is further supported by disease progression observed in the commercial orchard in Sutter County. The external symptoms found on first-leaf trees were masked by subsequent growth, leaving trees that appeared to be disease-free. Symptoms reappeared in 1981, which was the first full crop year, and the stress of nut production may have triggered symptom expression. The initial mechanical harvest also occurred in 1981 after mapping and sample collection was completed. The machine was new and had not been used previously, thus the mechanical harvester was not the agent of transmission. Had the unusual circumstance of symptom expression during the first leaf gone unnoticed, these trees would have been deemed healthy when they were fourth- through sixth-leaf trees and eligible as sources of commercial budwood. Other apparently healthy trees may have developed only internal symptoms or were symptomless carriers and would also be candidate source trees. Budwood collected from such trees before they are recognized as carriers could introduce deep bark canker into an orchard. Further dissemination of the pathogen by mechanical harvesting equipment and sap-sucking birds would follow. That stress plays a role in external

symptom expression further complicates the problem of identifying disease-free trees for propagation.

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