

**Evidence for Systemic Movement of  
*Erwinia rubrifaciens* in Persian Walnuts  
by the Use of Double-Antibiotic Markers**

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**ABSTRACT**

Extensive systemic spread of *Erwinia rubrifaciens* was observed in Persian walnut cultivar 'Hartley'. A mutant of the bacterium possessing antibiotic resistance markers for neomycin and rifampicin was used in the studies to monitor systemic spread. The bacteria spread at least 300 cm beyond the site of inoculation and remain latent in the distant symptomless regions. Phytopathology 63:1085-1086.

*Additional key words:* rifampicin, neomycin, mutant.

*Erwinia rubrifaciens* Wilson et al. (5) causes deep canker in several cultivars of Persian walnut with the commercially desirable 'Hartley' cultivar being most susceptible. One of the proposed measures of disease eradication is the surgical removal of cankers (4). If the bacteria move systemically beyond the region where canker symptoms are visible, surgical removal will not be effective. To test whether or not bacteria migrate or are transported into areas distant from the infection site and cankered regions, a sensitive assay method of detecting the bacteria is required. To attain this objective, we constructed a mutant of *E. rubrifaciens* carrying two drug-resistant markers. The use of bacteria with two genetic markers (rather than one) virtually eliminates the possibility of erroneously detecting spontaneous mutants in the isolation procedure.

A mutant of *E. rubrifaciens* with virulence equivalent to the parental wild type resistant to rifampicin and neomycin was constructed by conventional selective plating methods (1), without the use of mutagenic reagents. Walnut trees (*Juglans*

*regia* L. cultivar 'Hartley') (15 years old) were inoculated as described previously (2) with  $2 \times 10^8$  cells of *E. rubrifaciens* (mutant 6D328-10 Neo<sup>R</sup>, Rif<sup>R</sup>, Vir<sup>+</sup>) at various times of the year. At various times after inoculation, limbs were sawed off and brought to the laboratory for isolation. The outer bark of the limbs was swabbed with 90% ethanol and, thereafter, stripped with a sterile draw knife, and pieces of inner phloem-cambium tissue, ca. 1 × 2 inch, were removed at intervals along the limb. The pieces of phloem-cambium tissue were placed on the isolation medium: yeast extract-dextrose-calcium carbonate (YDC) agar containing 80 µg/ml each of rifampicin (Calbiochem, La Jolla, Calif.) and neomycin (Sigma Chemical Co., St. Louis, Mo.). Fungal growth was suppressed by incorporation of 250 µg/ml cycloheximide (Sigma) into the medium. Two precautions were always followed in the isolation procedure: (i) cuts were made sequentially from the distal to the proximal end of the limbs; (ii) dissecting instruments were sterilized after each cut. Plates were incubated for 24 hr at 30 C and the tissue pieces were removed to avoid inhibition of bacterial growth by substances (possibly oxidized phenolics) which diffuse from the woody tissue into the agar. Plates were incubated for 7 days at 30 C and any bacterial colonies that arose were transferred to fresh isolation plates. As expected, only bacteria resistant to rifampicin and neomycin grew. These bacteria were identified as the *E. rubrifaciens* mutant 6D328-10 Neo<sup>R</sup>, Rif<sup>R</sup> by their genetic markers, characteristic diffusible red pigment, and colony type always associated with these bacteria.

Isolations made in November and January, 2 and 5 months after inoculation, indicated that *E. rubrifaciens* moved beyond the point of the cankered or necrotic region associated with the inoculation site (Table 1). Isolations made during June, 3 to 4 months after inoculation, indicated that *E. rubrifaciens* was present even though necrosis did not occur (Table 1). The reasons for trees remaining healthy when inoculated in January or early February are unknown. The results are consistent with the observation that trees are not susceptible when inoculated in January (2). Our results show systemic transport or movement of the pathogenic bacteria in a relatively short time and fit well with our earlier field observations where cankers occurred along the limbs distant from the primary site of infection. The exact tissue in which the bacteria spreads is uncertain. The nonfunctional phloem may be the tissue in which bacteria translocate. The sieve plate pores of phloem cells are large enough in diameter to allow bacterial passage (3). Earlier observations by Schaad & Wilson (3) showed bacteria moved at least 90 mm beyond

TABLE 1. Recovery of neomycin-rifampicin-resistant *Erwinia rubrifaciens* from phloem-cambium tissue of walnuts

Date of		Length of necrotic canker from site of inoculation <sup>a</sup> (cm)	Distance bacteria recovered from site of inoculation (cm)
Inoculation	Isolation		
20 June	3 Nov.	25	304
25 Aug.	3 Jan.	10	228
25 Jan.	14 June	0	60 <sup>b</sup>
10 Feb.	14 June	0	105

<sup>a</sup> 0 indicates no visible necrosis or canker symptoms. Tree remained healthy.

<sup>b</sup> In this experiment, *Erwinia rubrifaciens* was recovered only from regions around 60 cm. Bacteria were not recoverable elsewhere, including the inoculation site.

visible symptoms. Our studies show that *E. rubrifaciens* spreads greater distances, at least 300 cm. The reasons why cankers are not produced contiguous with bacterial spread is not understood. It may be that the organism needs to be induced or be of sufficient population density to produce toxic substances.

Our procedure for qualitative detection of pathogenic bacteria by the use of double-genetic markers may be applicable to other bacterial diseases, especially those of tree crops. The procedure could be modified to quantitate the number of bacteria in various host plant regions. This was not feasible with this disease because of inhibitors emitted by assayed walnut tissues.

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