Systemic Spread of *Agrobacterium tumefaciens* Biovar 3 in the Vascular System of Grapes

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ABSTRACT

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The systemic movement of *Agrobacterium tumefaciens* biovar 3 was monitored with a mutant that was resistant to both streptomycin and rifampicin. Movement during the course of the experiments was confined exclusively to the xylem vessels. The bacteria moved rapidly 30 cm from the base of cut grape shoots. Additional evidence for long-distance vascular movement of *A. tumefaciens* was the development of tumors on aseptically wounded stem tissue 24 cm from the site of inoculated root tissue.

Crown gall caused by *Agrobacterium tumefaciens* (Smith and Townsend) Conn is a common disease of grapes (*Vitis* sp. L.) in many regions of the world. The pathogen induces gall formation on the roots; however, it more seriously affects the aerial parts, often girdling the trunk and killing cold-sensitive cultivars (3). Panagopoulos and associates (14,15) in Greece, Sule (23) in Hungary, and recently Burr and associates (4,5) in the United States have demonstrated that biovar 3 isolates are the predominant *A. tumefaciens* type infecting grape vines. The probability that *A. tumefaciens* travels as single cells through the xylem of the host was suggested initially by Riker (16). The rate of movement of *A. tumefaciens* cells in tomato plants was found to be 2 mm/hr (8). The translocation of crown gall bacteria in the plant is supported by the work of Stapp et al (21), who isolated the pathogen from the vessels of *Datura tatula* at a distance of 100 cm from the primary tumor.

Lehoczky (9–11) detected the movement of crown gall bacteria in xylem exudate of diseased vines, ostensibly tumor-free vines, and in grape root systems. He contended that *A. tumefaciens* could move long distances in grapes through the vascular system, a conclusion reached by El-Khalifa et al (7), who demonstrated that *A. tumefaciens* could move long distances from the point of inoculation and form secondary tumors in castor bean. Recently, Burr and associates (4,5) presented evidence on the systemic survival of *A. tumefaciens* in grapevine. However, no completely satisfactory explanation has been given for the manner by which secondary tumors are formed. Smith and associates (19,20) and Riker (16) reported that secondary tumors are formed from long strands (tumor strands) of cells that have undergone limited cell divisions and expansion. These studies suggested that secondary tumors were the consequence of metastasis of tumor cells. However, other investigators were unable to confirm this finding. Riker (17) and Suit and Eardley (22) demonstrated that bacteria per se are the causative agent of secondary tumors. In grapes, secondary tumors are found naturally on the main cane to a considerable height, often completely girdling it. Chamberlain (6) demonstrated that early-spring frosts result in massive cell injury along the vertical axis of the trunk in cold-sensitive cultivars of grapes. Abundant precipitation and sudden warming start a strong circulation of the vessel fluids that apparently carry the pathogen to the sites of low-temperature injury where infection ensues. Similarly, Lehoczky (9,11) suggested that under moist conditions in the spring, root pressure causes xylem fluids to sweep cells of *A. tumefaciens* from the root system toward internal wounds caused by low-temperature injury, where tumors then are formed.

This study was initiated to determine whether *A. tumefaciens* biovar 3 cells inoculated into grape roots can travel to the aerial parts of the vine and cause tumor formation there at an aseptically inflicted wound site.

MATERIALS AND METHODS

Bacterial strains and their cultivation. *A. tumefaciens* biovar 3 strains were routinely maintained on YTSA (yeast extract, 5 g; tryptone, 5 g; sucrose, 50 g; K_2HPO_4, 2 g; agar, 15 g; 1,000 ml of distilled water, pH 6.8) and grown at 27 ± 1 C. The three representative strains of biovar 3—Ag63, Ag57, and Ag123—isolated by Panagopoulos and Psallidas (14) that were used in these experiments were virulent on grapes. The virulence of a strain was indicated by gall formation at the point of inoculation of a susceptible grape cultivar.

Plant material. Throughout the study, two cultivars of grapes were used: Chancellor (*V. vinifera* × *V. rupestris*) and Catawba (*V. labrusca*), which are regarded as very sensitive and minimally sensitive, respectively, based on the size of the tumor they develop in response to natural and artificial inoculations (*unpublished data*).

Selection of mutants resistant to streptomycin and rifampicin. To study the transport of *A. tumefaciens* biovar 3 in grapevine tissue and the basis for the formation of secondary tumors, a mutant resistant to streptomycin and rifampicin (SR^r) was selected by the following method. Strains Ag63, Ag57, and Ag123 were grown to approximately 2×10^8 cells per milliliter in broth medium (YTSB) that contained streptomycin sulfate at 1,000 μg/ml. The antibiotics were added aseptically to the autoclaved, cooled YTSB. All inoculated flasks were shaken at 26 C for 48 hr. After incubation, 0.1 ml from each flask was spread onto each of three plates of YTSA that contained streptomycin and the plates were incubated at 26 C for 48 hr. Bacteria from a single colony growing on a plate containing streptomycin at 1,000 μg/ml were inoculated into YTSB containing rifampicin at 10 μg/ml and incubated at 26 C for 48 hr. Bacterial growth from a single colony was streaked on YTSA plates containing both antibiotics and incubated at 26 C for 48 hr. Colonies were selected from streptomycin-rifampicin agar plates after a 48-hr incubation. Virulence of the SR^r mutants was tested by stab-inoculating a heavy smear of 48-hr-old culture from a single colony with a sterile needle into surface-sterilized stems of

tomato (*Lycopersicon esculentum* Mill.) cultivar Rehovot 13 and grape cultivar Chancellor. Five plants of each host were inoculated in the greenhouse, and pathogenicity readings were recorded after 3 wk.

Inoculation studies. To study the long-distance transport of SR^r mutant in vascular systems of grapes, the Riker (16) technique was followed with slight modifications. Two series of 20 shoots, 30 cm in length and 5 mm in basal diameter, of both cultivars Chancellor and Catawba were used. Shoots were inoculated by submerging 4–5 cm of the freshly cut basal ends in a bacterial suspension (10^9 cells per milliliter). After 1.5 hr, shoots were transferred to tubes containing sterilized distilled water and left overnight in a growth chamber at 26 C. The basal cut of control shoots was submerged in sterile distilled water. At the end of a 24-hr period, the basal segment of each shoot that had been submerged in inoculum or sterile water was carefully excised to preclude further contamination of external surfaces. Then 1.0-cm segments were taken from each shoot at 10, 15, 25, and 30 cm above their cut bases. The segments were dipped in 70% ethanol, flamed, and then placed vertically on YTSA plates containing streptomycin and rifampicin. All specimens were prepared for light and scanning microscopy as described below.

Light microscopy. Stem segments (5–10 mm) were fixed in formalin-acetic acid-alcohol solution (FAA) (18) for 12 hr and then dehydrated in an ethanol series (20, 40, 60, 80, 100%), 20 min in each. They were then infiltrated for 12 hr and embedded using a JB-4 Embedding Kit (Polysciences, Warrington, PA). Sections 8–10 μm in thickness were cut with a rotary microtome using a glass knife, and sections were stained with 1% toluidine blue.

Scanning electronmicroscopy. Segments (5–10 mm) of inoculated and control shoots were fixed in 2.5% glutaraldehyde + 2% paraformaldehyde in 0.1 M sodium cacodylate buffer, 6.8 pH, at 4 C for 4 hr. The specimens then were rinsed in three changes of the same buffer and left in buffer overnight. The specimens were rinsed once in cold buffer for 30 min and transferred to cold 1% OsO_4 in the same buffer for 4 hr or overnight at 4 C. The specimens were then washed several times with cold buffer; cut into approximately 200-μm-thick sections on a Sorvall TC-2 tissue sectioner; dehydrated in a graded series of ethanol-water, 20, 40, 80, 3× 100% each for 20 min; and dried by the critical point method (1) in a Palaron SPC-1500 critical-point dryer. The specimens were mounted on aluminum stubs with conductive copper tape, sputter-coated with gold/palladium in a Palaron E5100 SEM coating unit, and examined in a JOEL JSM-35 scanning electron microscope at 20 kV.

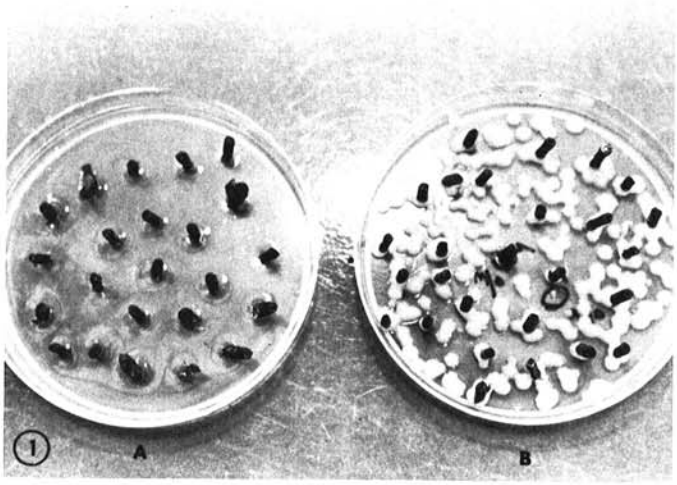
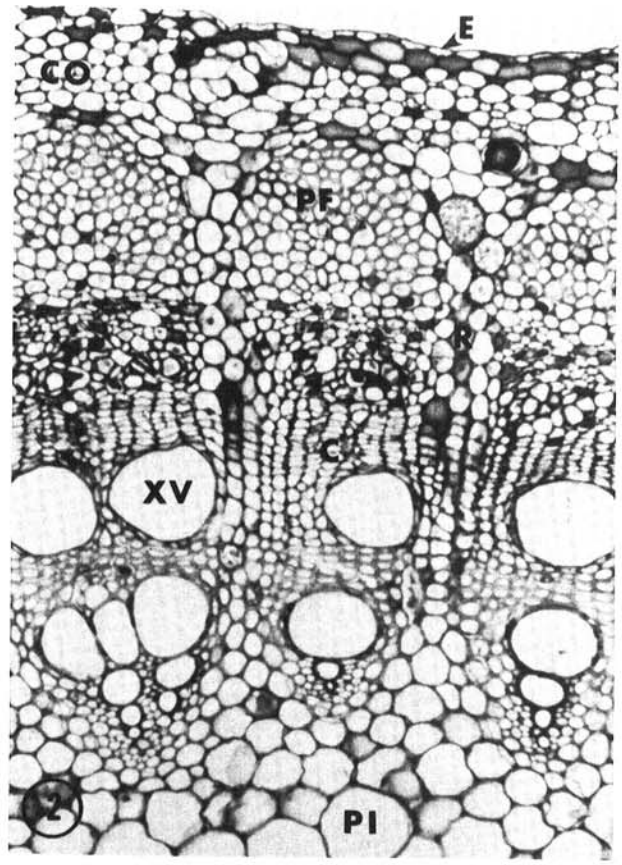
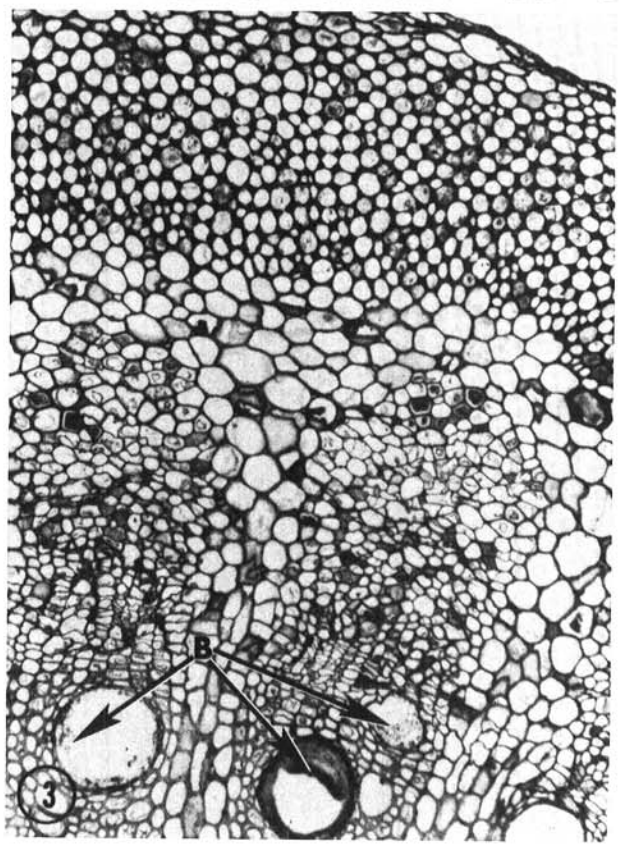
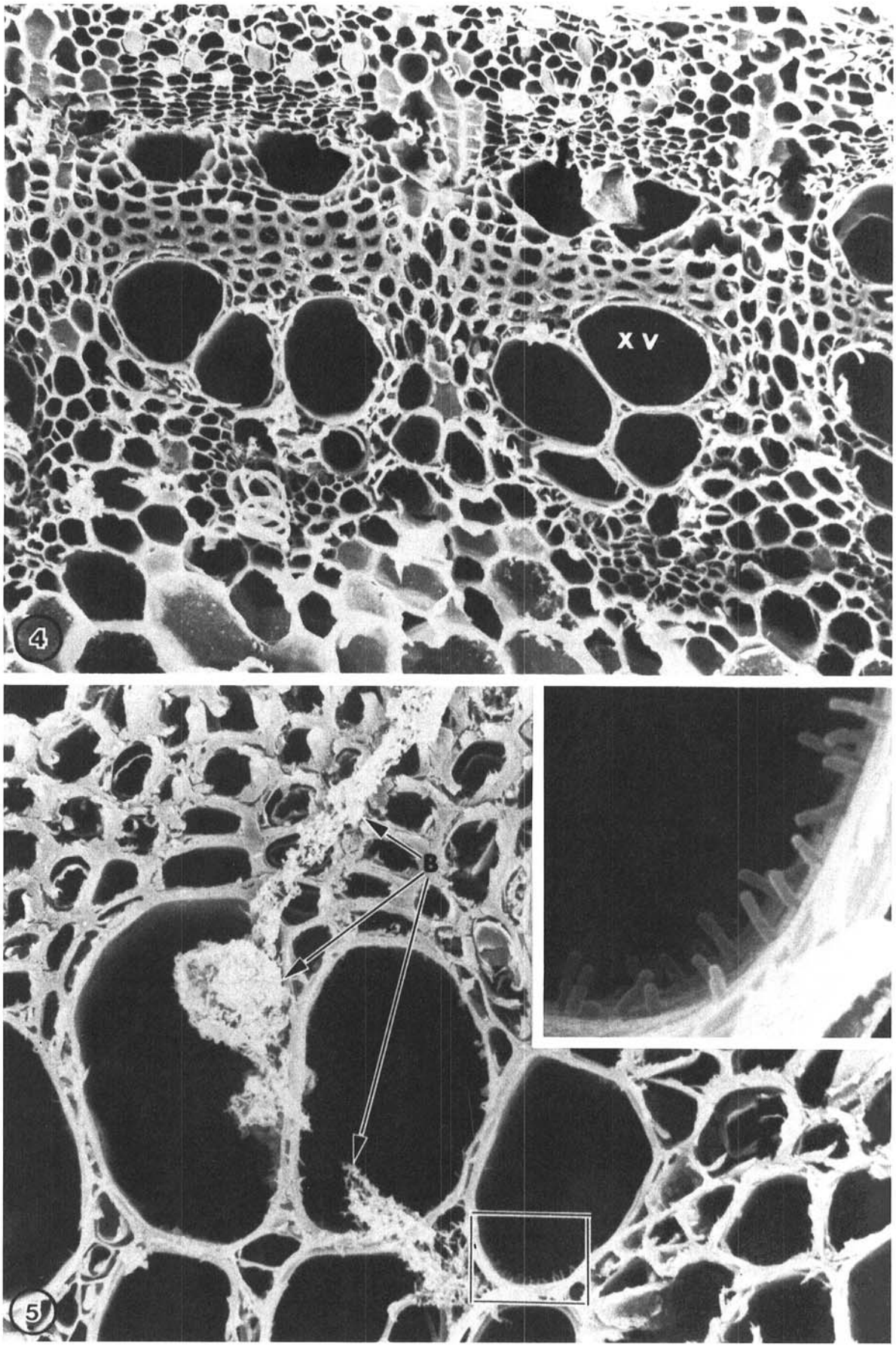


Fig. 1. Recovery of streptomycin-rifampicin-resistant mutant of *Agrobacterium tumefaciens* biovar 3 on antibiotic-containing medium at 10, 15, 25, and 30 cm from point of cut bases 24 hr after being immersed either in inoculum **(B)** or sterile water **(A)**. The four or five stem segments for each distance (in plate B) each have bacterial growth surrounding base. (Colonies developing away from stem sections reflect migration by means of flagella by some bacterial cells on surface of agar.)

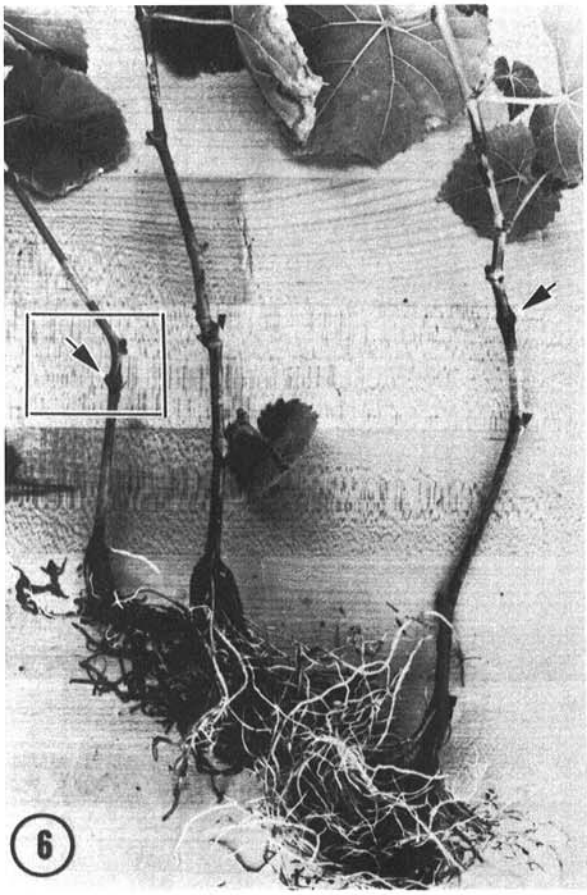


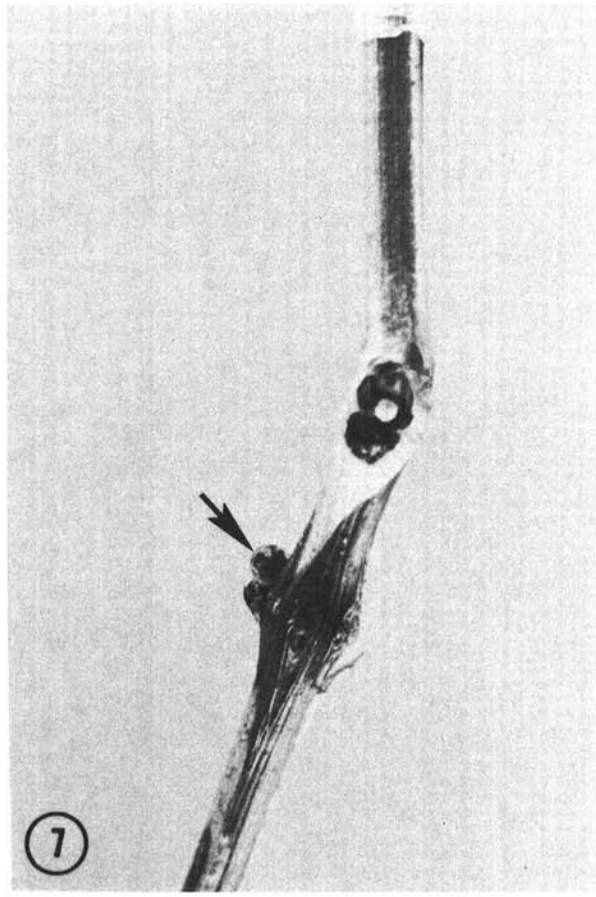


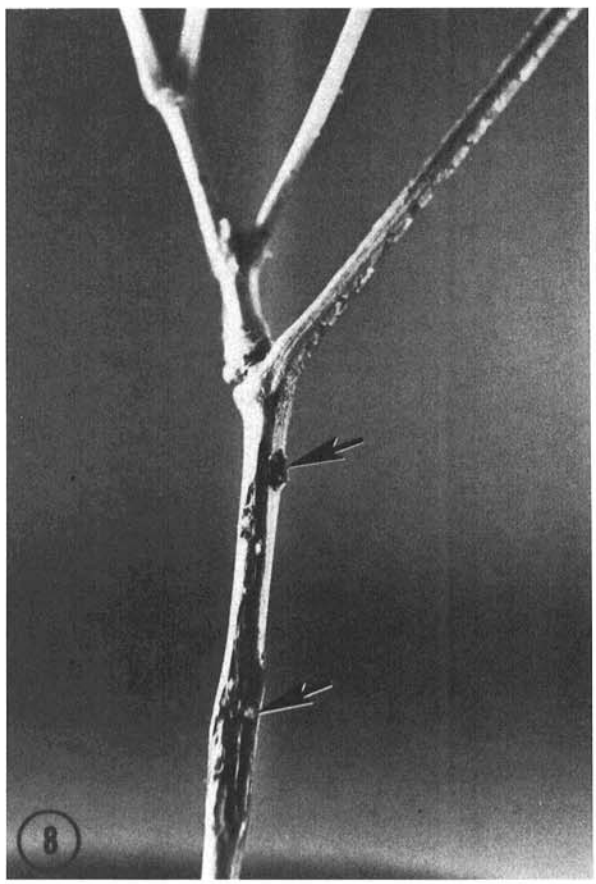
Figs. 2–3. Cross section **(2)** through uninoculated grape-shoot tissue revealing primary morphological features (E = epidermis, CO = cortex, PF = phloem fibers, R = ray cells, C = cambium, XV = xylem vessels, and PI = pith) (×400). Cross section **(3)** of grape shoot at 30 cm from point of entry of streptomycin-rifampicin-resistant mutant of *Agrobacterium tumefaciens* biovar 3. Xylem vessels of vascular bundle were only cells containing masses of bacteria (B) 24 hr after inoculation (×400).



Figs. 4–5. Scanning electron micrographs of transverse sections of grape shoots. **(4)** Uninoculated grape shoot, showing empty xylem vessels (XV) (×810). **(5)** Inoculated grape shoot 24 hr after inoculation with streptomycin-rifampicin-resistant mutant of *Agrobacterium tumefaciens* biovar 3 at 28 cm from point of introduction of bacteria. Bacterial clusters (B) were primarily in xylem vessels, strandlike formations of some bacterial cells believed to be artifacts of sample preparation (×910). (Insert) Individual bacterial cells appeared to become oriented and attached in polar fashion to inner xylem wall (×5,460).







Figs. 6–9. Tumor formation on Chancellor and Catawba grape cultivars after inoculation of their roots with streptomycin-rifampicin-resistant mutant of *Agrobacterium tumefaciens* biovar 3. **(6)** Secondary tumors developed at wound sites on stems of root-inoculated Chancellor plants (arrows). **(7)** Enlargement of area in Figure 6. **(8)** Small tumors (arrows) developed on stems of less sensitive Catawba cultivar. **(9)** Wounded (arrow) uninoculated control.

Tumor formation. Fifteen rooted cuttings of Chancellor and Catawba were either inoculated by soaking their roots in a suspension of 48-hr-old culture of SR^r at 10^9 cells per milliliter or in tap water for 24 hr, after which they were planted in pots containing autoclaved soil. All plants were kept in the greenhouse at 27 ± 5 C. After 2 days, wounds were made aseptically into vascular bundles by stabbing a sterile needle into surface-sterilized stems at different distances from the soil line of each plant. Wounded areas of inoculated and control plants were wrapped with Parafilm, and tumorigenesis readings were taken after 4–8 wk. Galls that formed were removed, washed in running tap water, cut with sterile scalpel into small pieces, and macerated in 2–3 ml of sterile water with mortar and pestle. A loopful of suspension was streaked on agar plates containing the two antibiotics. Cultures were incubated 2–3 days at 28 C and their virulence was established in grape and tomato stem tissue.

RESULTS

Isolation of antibiotic-resistant mutants and their translocation. After 48 hr of incubation at 26 C, strain Ag63 produced colonies that grew on plates containing streptomycin at 1,000 μg/ml and rifampicin at 10 μg/ml. However, neither Ag57 nor Ag123 was able to grow at these concentrations. Colonies of Ag63 that grew following three consecutive transfers on antibiotic-containing medium were regarded as antibiotic-resistant mutants. The Ag63 mutant strain was found to be virulent, as indicated by its ability to induce tumors on test plants.

Data recorded on the translocation of the mutant strain in grape shoots (Fig. 1) showed that the bacteria were readily recovered from all inoculated shoots of both cultivars. Despite the difference in sensitivity (based on tumor size) of the two cultivars to the crown gall pathogen, no difference in their translocation within the grape plant was noticed. Bacteria moved long distances in a short time, being translocated up to 30 cm within 24 hr from the point of entry.

Light and scanning electron microscopy. Further evidence of the long-distance translocation of *A. tumefaciens* biovar 3 in the vascular systems of grapes was obtained by light and scanning microscopy. The bacteria were present up to 30 cm from the inoculation point. Xylem vessels of the vascular bundles were the only cells containing masses of bacteria (Figs. 2 and 3). Scanning microscopy verified the presence of clusters of bacteria in the lumen of xylem vessels (Figs. 4 and 5). Bacteria were frequently in contact with the xylem wall surface and appeared to become oriented and attached in a polar fashion (Fig. 5). Bacteria were confined to the xylem vessels in both the sensitive and less sensitive cultivars during the 24-hr course of the experiment.

Tumor formation. Tumors on both cultivars were detected 3–5 wk after root inoculations. Tumors were found on aseptically wounded stems as far as 20–24 cm from the roots, and they were confined to the wounded sites. Twelve of 15 inoculated Chancellor plants developed tumors (Table 1). On the other hand, only 4 of 15 inoculated Catawba plants showed tumors. However, these tumors were small (2–3 mm) in diameter and remained so for the duration of the experiment. Tumors on Chancellor were larger in size (5–7 mm) and continued to increase in size during the 8-wk experimental period (Figs. 6–9). The SR^r mutant of Ag63 was easily recovered from all tumors that formed on both grape cultivars.

TABLE 1. Tumor formation on grapes after root inoculation with streptomycin-rifampicin-resistant mutant of *Agrobacterium tumefaciens* biovar 3

Cultivar	No. of plants inoculated[a]	No. of plants with tumors	Tumor size (mm)
Chancellor	15	12	5–7
Catawba	15	4	2–3
Controls[b]	30	0	...

[a] Plants were inoculated by dipping roots in inoculum suspension of streptomycin-rifampicin-resistant mutant Ag63 for 24 hr; subsequently, wounds were made aseptically on stems.
[b] Roots of 15 plants were dipped in sterile water.

DISCUSSION

The study of the movement of *A. tumefaciens* biovar 3 in grape tissue was facilitated by using the antibiotic-resistant mutant of Ag63. The mutant maintained its tumor-inducing ability, indicating that the mutation conferring resistance to antibiotics did not perceptibly alter virulence. Clearly, the recovery of the doubly marked *A. tumefaciens* at 30 cm from the point of entry of the bacteria indicated that *A. tumefaciens* biovar 3 can move through the vascular system of the inoculated grape shoots (Fig. 1). Visual evidence of systemic translocation of *A. tumefaciens* in the vascular system was obtained from light and scanning electron microscope observations. Bacteria were confined to the xylem vessels (Figs. 2–5). This finding is in agreement with previous reports by Riker (17), Hill (8), and Stapp et al (21), who demonstrated that *A. tumefaciens* can move long distances through the xylem vessels of other plants. Bacteria were readily recovered from both inoculated cultivars, Chancellor and Catawba, which are sensitive and less sensitive to crown gall, respectively. However, differences regarding their translocation in the xylem were not detected. This finding might be explained, at least partially, by the fact that no difference in the anatomy of the xylem, size, and number of vessels of both cultivars could be detected (*data not shown*). Additional evidence for long-distance vascular movement of *A. tumefaciens* in the grape plant was the development of secondary tumors 20–24 cm away from the roots where the bacteria were introduced, which agrees with the principle of bacterial transport in xylem vessels established for *Pseudomonas savastanoi* (E.F. Smith) Stevens in oleander (26), *P. caryophylli* (Burkh.) Starr et Burkh. in carnation (13), *Erwinia amylovora* in apple (12), and *A. tumefaciens* in grape (4,5,9,10,24). However, these data establish for the first time the movement of bacteria from root to aerial portions of the plant.

Recovery of the streptomycin-rifampicin-resistant mutant from tumors formed at aseptically wounded sites on grape plants inoculated through their roots leaves little doubt that bacteria per se are responsible for the initiation of these tumors. The foregoing results support the contention that secondary tumors on grapes are bacterially incited and that they are not bacteria-free, as proposed earlier by Braun and White (2). It is likely that the earlier report reflects the rapid death of large numbers of the *A. tumefaciens* population soon after transformation is accomplished (25).

It can be surmised from our experimental procedures that crown gall bacteria move in xylem vessels from root inoculation sites to stems where stab wounds rupture vessels, adjacent xylem parenchyma, and ray cells (25). The xylem parenchyma and ray cells are transformed and develop tumors much as they do in the field in spring following low-temperature injury coupled with soil-moisture conditions favoring root pressure, which forces bacteria-laden xylem fluids into the trunks.

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