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Permalink
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Journal
International Organization of Citrus Virologists Conference Proceedings (1957-2010), 8(8)

ISSN
2313-5123

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Publication Date
1980

DOI
10.5070/C59w33200r

Peer reviewed

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Effect of Penicillin or Tetracycline Injections of Citrus Trees Affected by Greening Disease Under Field Conditions in Reunion Island

B. Aubert and J. M. Bové

In a companion paper (Bové *et al.*, 1980) we explained why the prokaryote associated with citrus greening disease is not a mycoplasma, and showed that greening-affected citrus seedlings, which had absorbed penicillin through their roots, developed an abundant root system and produced new, vigorous, symptomless shoots. On the basis of these results, as well as previous ultrastructural studies (Garnier and Bové, 1977) we concluded that the greening organism probably possesses a peptidoglycan-containing cell wall typical of gram negative organisms and is a member of the division Gracilicutes (Bové *et al.*, 1980). In this paper, we add further evidence to this conclusion by showing that, under orchard conditions, penicillin injections into trunks of greening-affected sweet orange trees result in remarkable tree recoveries similar to those achieved with tetracycline injections.

MATERIALS AND METHODS

Sweet orange trees. Trees injected were in a 1 ha orchard planted in 1971 on well drained, flat, sandy soil, located on the leeward side of the island where the oriental psylla (*Diaphorina citri* (Kuway.), is prevalent (altitude: 10m) (Etienne and Aubert, 1980). The annual rainfall is 900 mm and irrigation is applied from September to December. The orchard is kept under permanent sod; weeds are controlled under the trees by hand or with herbicides. Two kg of a 15-7-24 NPK mix per tree are applied annually. Soil analysis has shown that most elements are at the optimum level, except magnesium, which is in excess. The orchard was under private management until 1977 and relatively poorly kept because of the severity of greening and low yields. For the purpose of antibiotic experiments, the orchard was taken over by Institut de Recherches sur les Fruits et Agrumes (IRFA) and has received adequate care since.

The sweet orange varieties, grafted on Troyer citrange and planted 8 by 8 m, were Valencia late, Hamlin, sweet seedling, Pineapple, and Washington navel. The budwood came from Corsica and was free of known virus and virus-like diseases. In 1977, six years after planting, most trees showed severe symptoms of greening. Yearly outbreaks of the oriental psylla were noted at each flush. The presence of the greening organism was confirmed by electron microscopy of leaf samples and graft transmission of the disease to healthy Madam Vinous seedlings kept in insect-proof screenhouses.

Intensity of greening symptoms. The severity of greening was rated on a scale from 0 to 40, obtained by inspecting the upper and lower halves of the tree on all four quadrants and grading each from 0 (apparently healthy) to 5 (severe symptoms including extensive dieback) and adding the eight resulting grades.

Injections. In December 1977, three months after bloom, 12 groups of three orange trees were selected in the orchard for injection. The trees of a given trio were of the same variety, of similar size, and showed greening symptoms of similar intensity (fig. 1A). There were five trios of Valencia late, two of Hamlin, two of sweet seedling, two of Pineapple, and one of Washington navel (fig. 1). One tree in each trio was injected with water (C), a second with penicillin G (P) and a third with tetracycline-HCl (T). Trees other than those in the 12 trios were used for preliminary injection experiments.

The device for injecting trees is sketched in figure 2 and was developed by one of us (B.A.). It has been described elsewhere (Bové, 1978; Aubert, 1979). Two hard plastic 1.5-liter bottles were used per tree. Each bottle was filled with 1 liter of liquid and pressurized to 5.0 kg/cm^2 before connecting it to the tree. The 36 trees were each injected with the 2 liters of liquid over a two-day period. Uptake time averaged 1 hour 45 minutes with a range of 20 minutes to 4 hours. The first injection was carried out in December 1977 with water, penicillin G or tetracycline-HC1. The antibiotics were applied at 3 g/liter (6 g/tree). Only penicillin G was injected in June and September 1978 by the same methods, for a total of 18 g/tree compared to 6 g/tree for tetracycline-HC1.

The severity of greening symptoms was recorded in October 1977, before the injections, and in October 1978. Yields of individual trees were determined in March-April, 1978 and 1979.

Bioassays. The presence of antibiotics in the trees as a function of time after injection was determined by bioassays on *Bacillus subtilis* (ATCC 6633) for tetracycline, and on *Sarcina lutea* (ATCC 934) for penicillin according to the method of Grove and Randall (1955). The assays were performed on 200 mg (fresh weight) of leaf midribs, flowers, or rootlets, taken at random from the injected trees. Rootlets were surface sterilized in 1 per cent sodium hypochlorite for 10 seconds and rinsed three times in sterile water. After grinding the sample with 1 ml of water in a mortar, 20 μl of the homogenate was deposited on a 6-mm Whatman AA or Schleicher-Schull 2668 paper disk. For fruit near maturity the bioassays were made directly with 20 μl of fruit juice. The disks were placed on freshly inoculated culture medium. The diameters of the inhibition zones were read after 24 hours at 28° C and compared with those given by known amounts of penicillin G or tetracycline-HC1.

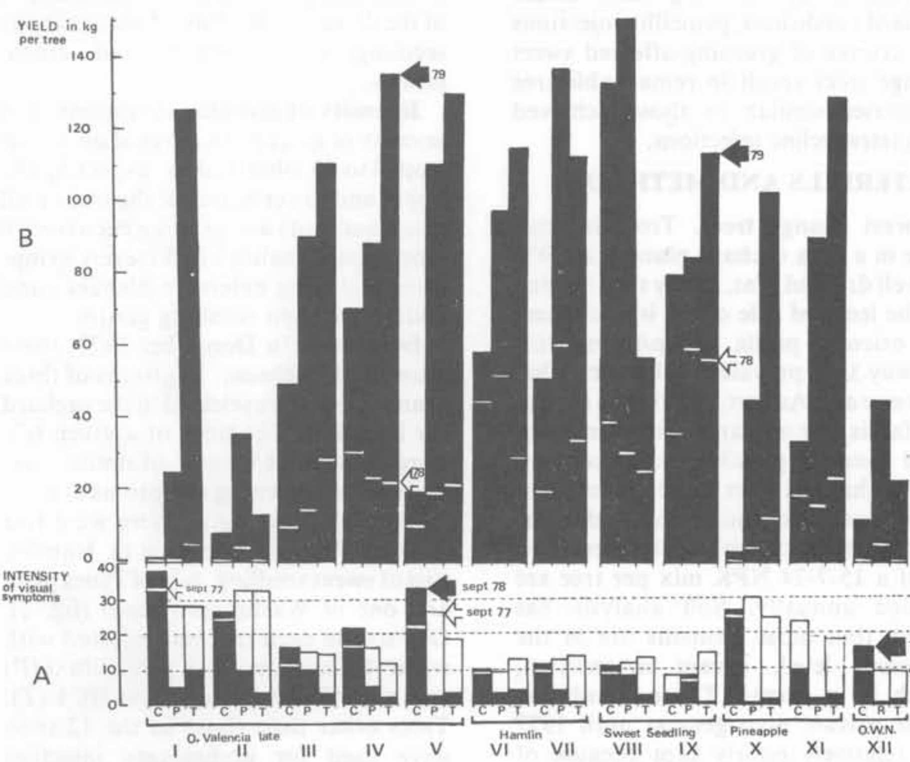

Fig. 1. Symptom intensity (A) and yield in kg/tree (B) of water, penicillin, or tetracycline injected sweet orange trees.

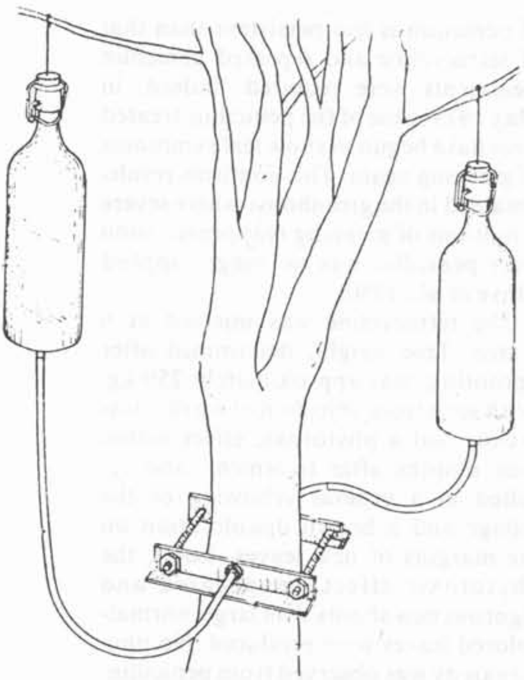

Fig. 2. Device for tree injection.

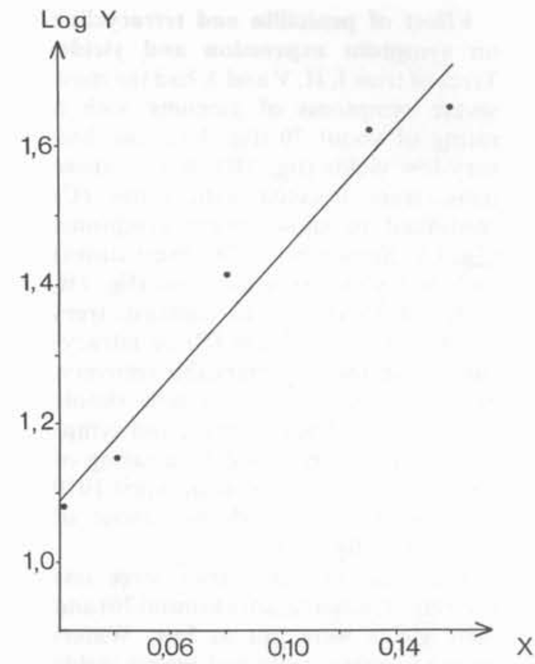

Fig. 3. Relation between penicillin concentration, X and diameter of inhibition zone, Y.

The relation between penicillin concentration, χ, given in international units (1 IU = 0.607 μg) and diameter of inhibition zone, y in cm, was found to be $\log y = 4\chi + 1$ by the least squares method (fig. 3).

Both penicillin G and tetracycline-HCl were obtained from SPECIA.

RESULTS

Bioassays. No inhibition zone was observed in any tissues of the 12 trees injected with water. Since this work was essentially initiated to study the effect of penicillin, more bioassays were conducted with leaf midribs from penicillin-injected trees than those from tetracycline-treated trees. The main purpose of the bioassays was to determine the uptake, distribution, and persistence of the antibiotic in the tree. Inhibition zones to penicillin or tetracycline could be seen with extracts from young leaf midribs harvested as early as 15 minutes after injection was begun. In one experiment, five Washington navel trees were injected with 0, 3, 6, 9, or 12 g of penicillin in a volume of 2 liters. It took 20 minutes for the liquid to enter the trees. A sixth tree received a paste of 15 g of dry penicillin in vaseline applied to six holes drilled in the trunk. Penicillin in the leaf midribs was estimated at various times after injection (fig. 4). No antibiotic could be detected at any time when trees were injected with water or when penicillin was applied in a paste. However, penicillin could be detected in the leaves at its highest concentration as early as 1 hour after the beginning of injection, when injected with a solution containing 6, 9, or 12 g of the compound. With 3 g, the highest concentration was detected 10 hours after injection. Penicillin remained at a high level for approximately 70 hours, then decreased, and could not be detected after 120 to 145 hours. In small stunted trees, penicillin (6 g/tree) was still present after 250 hours.

The antibiotic concentration in flowers followed approximately the same pattern as in leaves, but it was always 20 per cent lower in fruit juice. Penicillin could be demonstrated in the roots of some, but not all, trees.

Effect of penicillin and tetracycline on symptom expression and yields. Trees of trios I, II, V and X had the most severe symptoms of greening with a rating of about 30 (fig. 1A), and had very low yields (fig. 1B). Within these trios, trees injected with water (C) continued to show severe symptoms (fig. 1A, September 1978 observations) and their yields remained low (fig. 1B, April 1979 harvest). In contrast, trees injected with penicillin (P) or tetracycline (T) showed a remarkable recovery, produced many virogous new shoots with large expanded leaves, and symptom intensity decreased to a rating of about 5 (fig. 1A). Yields in April 1979 were greatly increased over those of April 1978 (fig. 1B).

The trees of other trios were less severely affected (grades around 10) and their yields were not as low. Water-injected control trees had higher yields in general in April 1979 than in 1978, which reflected the improved orchard care since 1977. The penicillin or tetracycline-injected trees produced vigorous, symptomless shoots and many trees appeared almost normal (grades below 5). They had much less fruit drop in December 1978. Their 1979 yields were far higher than those of 1978, and were also higher than the 1979 production of the water-injected control trees.

In summary, for the 12 trios the average 1979 production of the water-, penicillin-, and tetracycline-injected trees was 33, 76, and 99 kg/ tree, respectively. In addition to low yields, the water-injected trees had many small, lopsided fruits. Although the penicillin-injected trees were markedly improved in comparison with the water-injected controls, they remained inferior to the tetracycline-injected trees except in trios II, VII, VIII, and XII where the penicillin-treated trees had the highest yields.

The penicillin-treated trees have received three injections totaling 18 g (December 1977, June, and September 1978) while the tetracycline-treated trees were injected only once with 6 g (December 1977). The beneficial effect of penicillin is less persistent than that of tetracycline and repeated penicillin treatments were required. Indeed, in May 1979 some of the penicillin-treated trees have begun to show leaf symptoms of greening again. This confirms results obtained in the greenhouse where severe symptoms of greening reappeared soon after penicillin was no longer applied (Bové *et al.*, 1980).

The tetracycline was injected at 6 g/tree. Tree weight, determined after uprooting, was approximately 250 kg. With such trees, injection of 6 g of tetracycline had a phytotoxic effect within four months after treatment, and resulted in a general yellowing of the foliage and a brown discoloration on the margins of new leaves. Later, the phytotoxic effect disappeared and vigorous new shoots with large, normal-colored leaves were produced. No phytotoxicity was observed from penicillin.

DISCUSSION

Penicillin has a marked effect on greening-affected trees, inducing the formation of new, vigorous, symptomless growth. Fruit quality is improved and yields are significantly increased. The results obtained under field conditions with penicillin injected in the trunks confirm and extend those obtained in the greenhouse where penicillin was fed through the roots of young, greening-affected, sweet orange seedlings (Bové *et al.*, 1980). As discussed elsewhere (Bové *et al.*, 1980) on the basis of these data as well as on ultrastructural studies *in situ* (Garnier and Bové, 1977) the greening organism definitely is not a mycoplasma, but a prokaryote probably having a peptidoglycan-containing gram negative type of cell wall (Gracilicutes; see Gibbons and Murray, 1978).

Both penicillin and tetracycline are effective against greening. A single application of 6 g tetracycline per tree has a longer lasting effect than the same amount of penicillin. At this rate, tetracycline is phytotoxic, but penicillin is not.

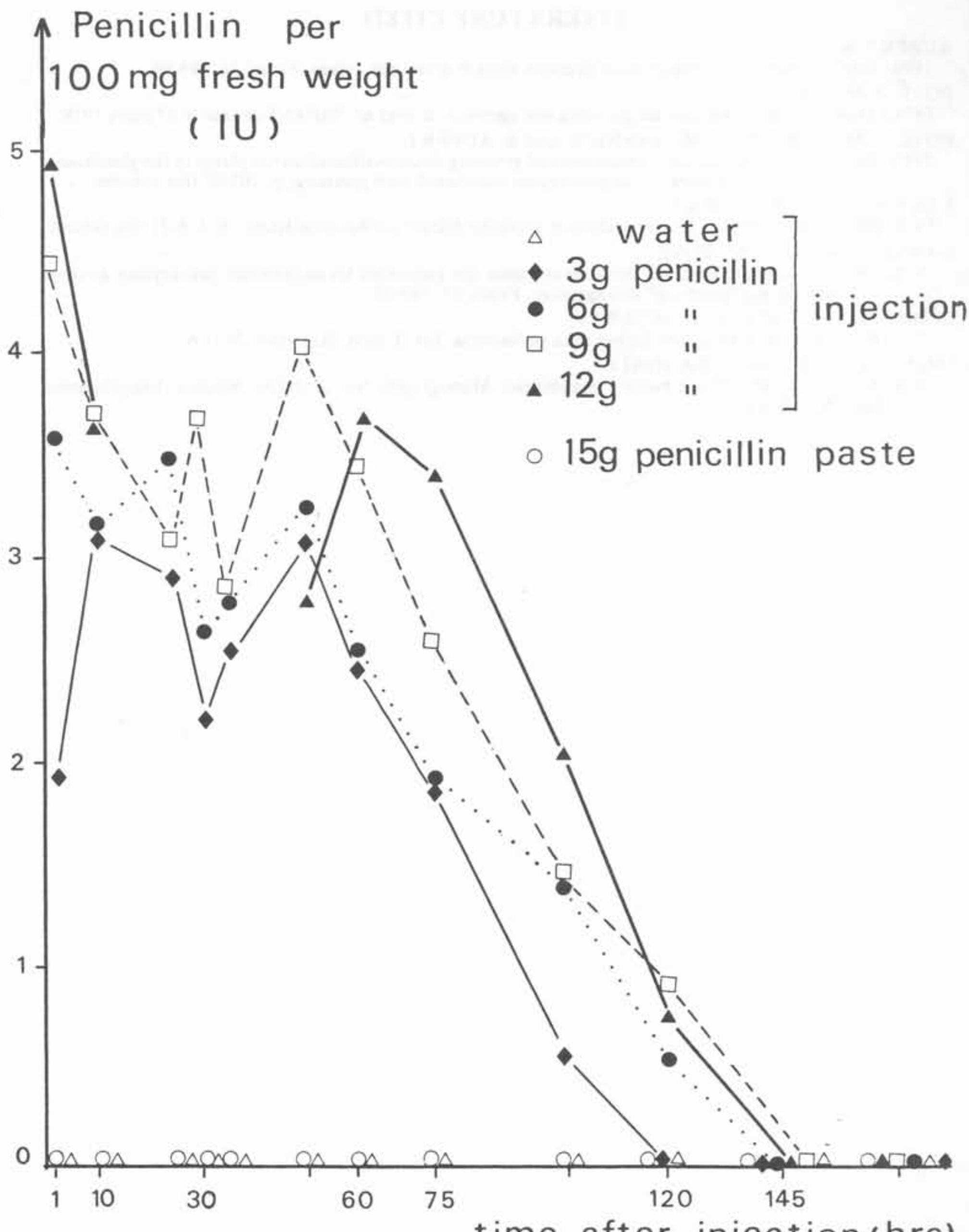

Fig. 4. Penicillin concentration in leaf midribs as a function of time after injection.

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