GREENING, STUBBORN AND RELATED DISEASES

Penicillin and Tetracycline Treatments of Greening Disease-affected Citrus Plants in the Glasshouse, and the Bacterial Nature of the Procaryote Associated with Greening

J. M. Bové, P. Bonnet, M. Garnier, and B. Aubert

DEDICATED TO RALPH E. SCHWARZ (1929 - 1979)

Since the discovery of the procaryote associated with citrus greening disease (Lafleche and Bové, 1970*a* and *b*), our group has established the following facts. 1) Under normal electron microscopy, the envelope surrounding the organism is comprised of three zones: a dark, electron-absorbing inner zone, a dark outer zone, and an intermediate, clear, electron transparent zone. The thickness of the three zones is approximately 250 Å. Such an envelope is far too thick to be a single unit membrane, and the mycoplasma nature of the greening organisms was questioned (Saglio *et al.*, 1971; Bové and Saglio, 1974). 2) The various geographical forms of greening (blotchy mottle, citrus decline, leaf mottling, yellow branch, and likubin), are all characterized by the same 250 Å-thick envelope system (Bové and Saglio, 1974; Garnier *et al.*, 1976). 3) While the inner and outer dark, electron-dense zones are often parallel, they are clearly separated, suggesting that each is a single membrane (Garnier *et al.*, 1976). 4) Each of the two electron-dense zones can be resolved into a triple-layered unit membrane, 90-100 Å thick (Garnier and Bové, 1977), confirming an earlier report by Moll and Martin (1974). The inner membrane is the cytoplasmic membrane, the outer membrane is a cell wall.

Recently, Gibbons and Murray (1978) have proposed that the kingdom Procaryotae be divided into four divisions according to the presence of peptidoglycan (PG) and the type of cell wall. PG is the specific constituent of most bacterial cell walls. The division Gracilicutes is comprised of organisms having a Gram-negative, membranous cell wall containing PG. The Firmacutes include the prokaryotes with a Gram-positive, PG-containing, nonmembranous cell wall. The Mendocutes have a cell wall, but no PG; halophilic bacteria belong to this division. The Mollicutes have no cell wall or PG; they are surrounded only by a cytoplasmic membrane, shared by all living cells.

The greening organism (GO) has a cell wall, and is clearly not a mollicute. With its membranous cell wall, the GO is obviously not a firmacute, but could belong to the Gracilicutes or to the Mendocutes. The typical triple-layered outer membane of the GO suggests its inclusion in the division Gracilicutes. If a gracilicute, the GO must contain PG. Hence, the presence or absence of PG would determine the classification of the GO. The PG of gracilicutes is generally seen as an electron-dense zone, sometimes called R layer, located between the cytoplasmic membrane and the membranous cell wall. Such a zone could not be observed in the GO (Moll and Martin, 1974; Tanaka and Doi, 1976). However, we have occasionally noticed that, at certain locations, the inner layer of the outer membrane is somewhat thicker, more electron-dense, than the other layers, reminiscent of the PG zone of certain gracilicutes (Garnier and Bové, 1977). Since the GO is not available in culture, direct biochemical detection of PG has not been achieved; however, indirect indications for the occurrence of PG in the GO can be obtained by studying the effect of penicillin on greening-affected citrus plants. This antibiotic inhibits a late step (transpeptidation) in the biosynthesis of PG (Ghuysen and Shockman, 1973).

In this paper, we report that greening-affected sweet orange plants outgrow symptoms when treated with penicillin G and that no GO's can be seen in the sieve tubes of new, symptomless leaves. However, the GO's and the symptoms reappear when the treatment is stopped. From these results, we conclude that the greening organism probably possesses PG and belongs to the division Gracilicutes. Preliminary reports of this work have already appeared (Bové, 1978; Garnier and Bové, 1978; Bové, 1979).

MATERIALS AND METHODS

In the first experiment, we used diseased citrus seedlings which had been infected with the greening pathogen for many years. Seedlings infected with *Spiroplasma citri* and the leaf curl pathogen were compared to greening-affected plants. The second experiment was carried out with healthy or greening-affected sweet orange buds propagated on healthy rootstocks. The effect of tetracycline-HCl as well as penicillin was investigated. Healthy and greening-infected Madam Vinous sweet orange seedlings were used in the third experiment.

All plants were grown in sterilized white sand in 5 (expt. III)- or 10 (expt. I and II)-liter plastic containers. Penicillin or tetracycline was applied in a nutrient solution (final pH: 7.2) devised for citrus by P. Smith (USDA, Orlando, Florida). The plants were kept in a glasshouse between 20 and 27°C, and illuminated for 16 hours with supplemental fluorescent light in winter. They were watered with nutrient solution (with or without antibiotics) once every 1 or 2 weeks, as needed. Sodium benzylpenicillin (Penicillin G) was from Specia, Paris, France and tetracycline-HCl from Pfizer, Amboise, France. The presence of penicillin or tetracycline in leaves of the treated plants was checked according to Aubert and Bové (1980) after each immersion of the roots into the antibiotic solution. The concentration of penicillin in the leaves was approximately one-hundredth (1/100) that of the solution used for root immersion. Electron microscopy techniques have been described earlier (Garnier and Bové, 1977).

RESULTS

Experiment I. The following plant material was used:

a. Healthy: two Hamlin and four Madam Vinous sweet orange seedlings.

b. Greening-affected: six Hamlin seedlings infected in 1971-1973 with likubin (PD-TY-10), six Hamlin seedlings infected in 1970-1974 with Indian greening (Poona), two Hamlin seedlings infected in 1970 with leaf mottling (Lipa G 3), two Hamlin seedlings infected in 1974 with Reunion greening, and four Madam Vinous seedlings infected in 1970 with South African greening (G 1 A S).

c. Leaf curl-affected: four Madam Vinous seedlings infected in 1972 with leaf curl (Campinas), two Orlando tangelo seedlings infected in 1975 with leaf curl (Campinas).

d. Stubborn affected: four Madam Vinous seedlings infected in 1970 with C159 stubborn (courtesy Prof. E. C. Calavan).

Half of each group of seedlings was treated with penicillin, and the other half left untreated as controls. The outline of the antibiotic treatment in expt. I is given in fig. 1 (expt. I).

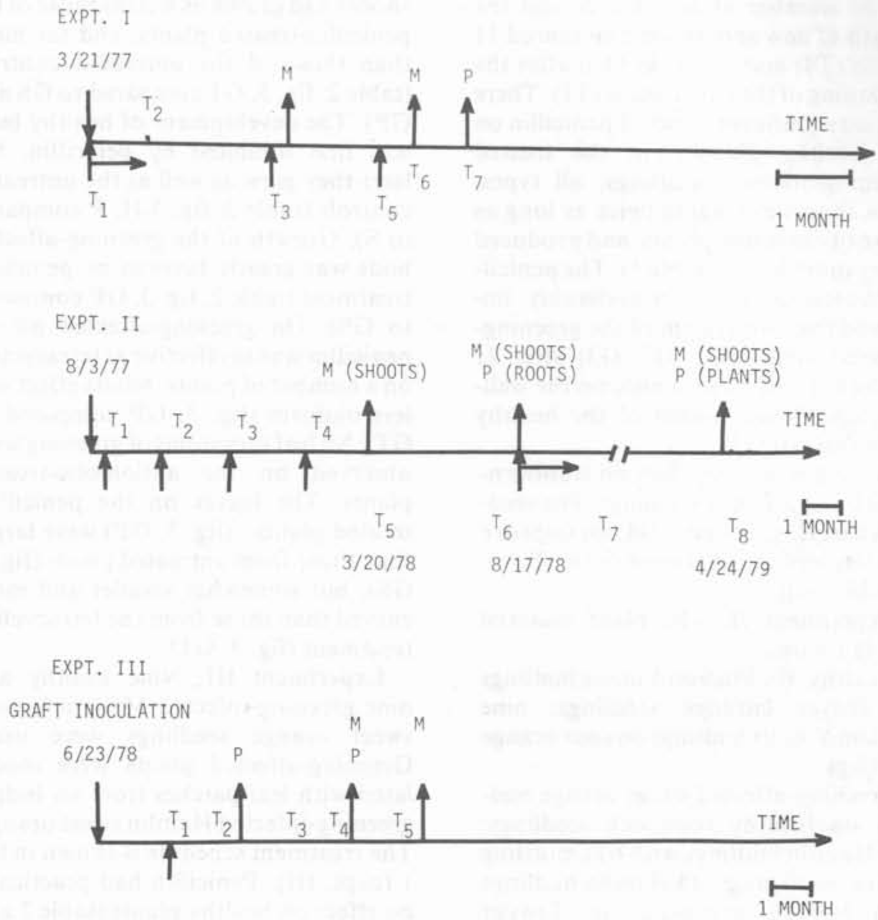

Fig. 1. Outline of antibiotic treatments — Expt. I. At T_1, T_3 and T_5 the roots of sweet orange seedlings were immersed for 17 hr in a nutrient solution containing penicillin-G (1 g/liter). From T_1 to T_2 the seedlings were watered with nutrient solution containing penicillin-G (0.2 g/liter). At T_4 (June 6, 1977) and T_6 (July 25, 1977) the number of new leaves and the length of new shoots were measured (M); at T_7 (August 17, 1977) the root system was photographed (P). Expt. II. Buds were propagated August 3, 1977 on seedlings in pots. At T_1, T_2, T_3, T_4, T_6 the pots were immersed in nutrient solution containing penicillin-G (1 g/liter) or tetracycline-HC1 (1 g/liter) for 17 hr. From T_6 to T_7 plants were watered with nutrient solution containing penicillin-G (0.2 g/liter) or tetracycline-HC1 (0.05 g/liter). Expt. III. From T_1 to T_3 plants were watered with nurtient solution containing penicillin-G (0.5 g/liter). At T_2 (October 20, 1978) and T_4 (January 25, 1979) photographs were taken (P). At T_4 and T_5 (March 27, 1979) length of shoots was measured (M). Control plants in all experiments were treated with nurtient solution only.

The number of new leaves and the length of new shoots were measured 11 weeks (T4) and 18 weeks (T6) after the beginning of the experiment (T1). There was no significant effect of penicillin on the healthy plants, but the treated greening-affected seedlings, all types, grew shoots averaging twice as long as those of untreated plants, and produced many more leaves (table 1). The penicillin treatment (+ P) considerably improved the root system of the greening-affected plants (G1, G2, G3) after 21 weeks but had no influence on the well-developed root system of the healthy plants (H) (fig. 2).

Penicillin had no effect on stubborn- or leaf curl-affected seedlings. The seedlings carrying leaf curl did not improve with tetracycline treatment either (Bové, unpublished).

Experiment II. The plant material was as follows:

Healthy: six Madam Vinous budlings on Troyer citrange seedlings; nine Madam Vinous budlings on sour orange seedlings.

Greening-affected sweet orange budlings on healthy rootstock seedlings: nine Hamlin budlings with leaf mottling on Troyer citrange; 15 Hamlin budlings with Indian greening on Troyer citrange; 15 Hamlin budlings with Indian greening on Rangpur lime; six Hamlin budlings with likubin on Troyer citrange.

One-third of each group of budlings was treated with the nutrient solution alone, one-third with penicillin, and one-third with tetracycline.

The treatment schedule is explained in fig. 1 (expt. II). Tetracycline was used at 1 g/liter and induced severe phytotoxicity, as indicated by leaf drop and root necrosis. The shoots produced from healthy buds on tetracycline-treated plants were, 21 months after budding, still shorter, on the average, than those from untreated or penicillin-treated plants (table 2, fig. 3-H).

The growth of greening-affected plants was at first also retarded by the tetracycline treatment (table 2). However, 21 months after budding, their shoots had grown as well as those of the penicillin-treated plants, and far more than those of the untreated controls (table 2; fig. 3, GT compared to GS and GP). The development of healthy buds was first inhibited by penicillin, but later they grew as well as the untreated controls (table 2; fig. 3-H, P compared to S). Growth of the greening-affected buds was greatly favored by penicillin treatment (table 2; fig. 3, GP compared to GS). On greening-affected plants, penicillin was as effective as tetracycline on a number of plants, but its effect was less uniform (fig. 3, GP compared to GT). No leaf symptoms of greening were observed on the antiobiotic-treated plants. The leaves on the penicillin-treated plants (fig. 3, GP) were larger than those from untreated plants (fig. 3, GS), but somewhat smaller and more curved than those from the tetracycline treatment (fig. 3, GT).

Experiment III: Nine healthy and nine greening-infected Madam Vinous sweet orange seedlings were used. Greening-affected plants were inoculated with leaf patches from an Indian greening-infected Hamlin sweet orange. The treatment schedule is shown in fig. 1 (expt. III). Penicillin had practically no effect on healthy plants (table 3 and fig. 4), but greatly improved the growth of greening-affected plants within 4 months. The improvement was strongly evident 7 months after inoculation (fig. 4, G2). No GO's could be detected in the symptomless leaves of these plants. In this experiment, the penicillin treatment was started 2 months after inoculation (fig. 1, expt. III, T1) and lasted for 3 months. At the end of the experiment (9 months postinoculation), penicillin treatment had been withheld for almost 4 months, and leaf symptoms of greening began to reappear on the penicillin-treated seedlings. Then, GO's could again be detected in the affected leaves.

DISCUSSION

When first described by Lafleche and Bové (1970*a*), the GO was called "mycoplasma-like." Soon thereafter the stubborn organism (SO) was discovered by Igwegbe and Calavan (1970), and their discovery was immediately confirmed by Lafleche and Bové (1970*b*). The GO and the SO were compared in sweet orange seedlings of the same cultivar and found to be morphologically different (Lafleche and Bové, 1970*b*; Saglio *et al.*, 1971). While the SO was surrounded by a simple unit membrane, the GO had an envelope at least twice as thick as that of the SO. The SO was truly mycoplasma-like, and further work, which culminated in the isolation, cultivation, and characterization of the SO, now called *Spiroplasma citri*, confirmed its mycoplasma nature (Fudl-Allah *et al.*, 1972; Bové *et al.*, 1973; Cole *et al.*, 1973; Saglio *et al.*, 1973; Bebear *et al.*, 1974). With an envelope far too elaborate to be a simple unit membrane, the GO could not be mycoplasma-like. This conclusion was stated as early as 1971 (Saglio *et al.*, 1971), reiterated several times (Saglio *et al.*, 1972; Bové and Saglio, 1974; Bové, 1975), amply documented and discussed in length (Garnier *et al.*, 1976), and confirmed by others (Chen *et al.*, 1972; Su and Leu, 1972; Moll and Martin, 1974; Tanaka and Doi, 1976). Furthermore, Moll and Martin (1974) presented evidence that the GO had a double-track membrane-like cell wall in addition to its inner cytoplasmic membrane. They correctly proposed the term "bacterium-like organism." However, since they could not detect a peptidoglycan layer (R layer) between the inner membrane and the outer membane-like cell wall, they thought the GO was not similar to Gram-negative bacteria. Garnier and Bové (1977) extended these observations, and clearly confirmed that the GO was surrounded by two triple-layered unit membranes. In addition, they observed sometimes, although rarely, that at certain locations the inner layer of the outer membrane was thicker than the other layers, a situation reminiscent of the gracilicutes such as *Escherichia coli.*

The present work showed that greening-affected plants treated with penicillin grew better, produced more roots and larger symptomless shoots and leaves than untreated controls. A beneficial effect of penicillin was also noted when penicillin was injected into the trunk of field-grown, greening-affected sweet orange trees in Reunion Island (Aubert and Bové, 1980). Schwarz *et al.* (1974) have also injected greening-affected Valencia sweet orange trees with penicillin or with different tetracyclines. However, all penicillin and some tetracycline treatments appeared ineffective, probably because dosages were too low for the large volume of the trees (Schwarz *et al.*, 1974). In Reunion, beneficial effects of penicillin were obtained with amounts 1,000 times higher. Su and Chang (1976) observed that shoots produced from likubin-affected citrus budwood dipped into penicillin G (120 mg/liter) produced healthy-looking shoots with moderate growth. However, they drew no conclusions as to the nature of the likubin pathogen from this positive penicillin effect, and continued to call it an MLO. T. Miyakawa (personal communication) has also observed a beneficial effect of penicillin-treated, likubin-affected citrus material.

From the data presented here and in the companion paper (Aubert and Bové, 1980) and that obtained by others (Su and Chang, 1976; Miyakawa, personal communication), penicillin clearly has a positive effect on greening-affected glasshouse- or field-grown sweet orange plants. The positive effect of penicillin on greening-affected plants is evidence for the presence of PG in the GO, and, hence, for the gracilicute-like nature of the GO for the following reasons:

1) The only known mode of action of penicillin is the inhibition of PG biosynthesis. Mollicutes, having no cell wall nor PG, should not be affected by penicillin. There are a few exceptions where penicillin G inhibits the growth of animal mycoplasmas such as *Mycoplasma neurolyticum* (Wright, 1967), *M. suipneumoniae* (Friis, 1971), and *M. dispar* (Andrews *et al.*, 1973). These inhibitory effects are not well understood, but in the case of *M. suipneu-*

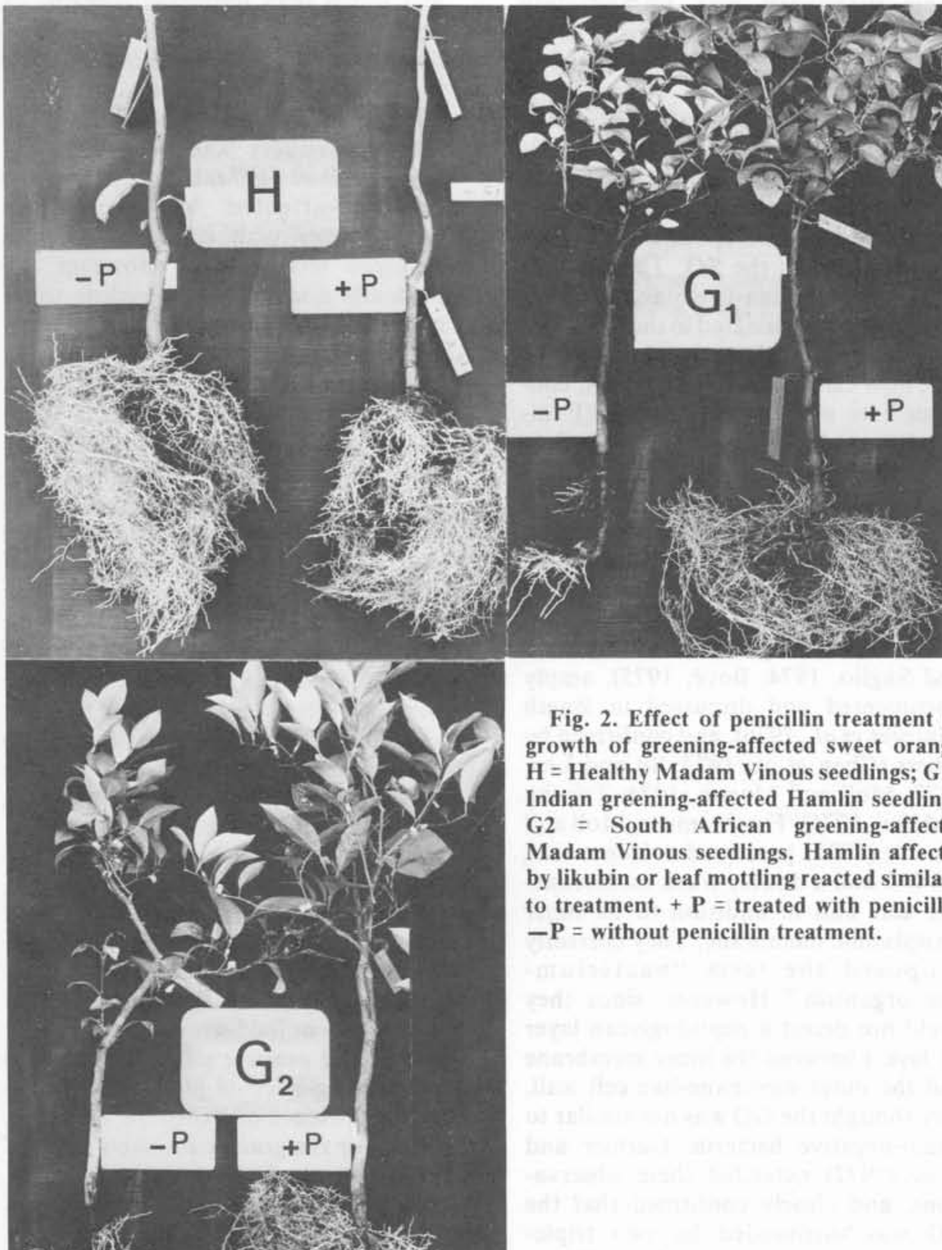

Fig. 2. Effect of penicillin treatment on growth of greening-affected sweet orange. H = Healthy Madam Vinous seedlings; G1 = Indian greening-affected Hamlin seedlings; G2 = South African greening-affected Madam Vinous seedlings. Hamlin affected by likubin or leaf mottling reacted similarly to treatment. + P = treated with penicillin; —P = without penicillin treatment.

moniae, the inhibition is apparently due to a toxic impurity in certain batches of the antibiotic (Whittlestone, 1979).

2) Penicillin has no effect when applied to citrus infected with the mycoplasma *S. citri* or to periwinkles infected with mycoplasma-like organisms (MLO) (Bové, 1979).

3) The organism associated with club leaf of clover is thought to be similar to the GO. Interestingly, penicillin treatment of clover club leaf plants also resulted in symptom remission (Windsor and Black, 1973).

4) The fact that no PG zone (R layer) is seen in the GO is not contradictory with the proposed gracilicute nature of the GO. In many gracilicutes, the PG layer is extremely thin (20° Å) and difficult to demonstrate. For instance, the Gram-negative bacterium *Pseudomonas aeruginosa* has an outer membrane, but the PG layer is too thin to be resolved (Costerton, 1979). Only a thin PG layer would be required for an organism such as the GO, which has to remain flexible and pleomorphic to move through the pores of the sieve tubes.

5) Penicillin mainly inhibits Gram-positive bacteria (Firmacutes), but inhibits some gracilicutes such as the Neisseriae and *Moraxella*. Hence, the proposed gracilicute nature of the GO and its sensitivity to penicillin are not contradictory.

6) With a triple-layered outer membrane as a cell wall and with the presence of PG, the GO fits the definition of the gracilicutes.

In conclusion, we propose the term "gracilicute-like bacterium" for the GO. This term is more precise than the "bacterium-like organism" (BLO) proposed by Moll and Martin (1974), and reveals recent research progress. The names "rickettsia-like organism" (RLO) or "rickettsia-like bacterium" (RLB) recently used (Hopkins, 1977; Tsai, 1979; Nienhaus and Sikora, 1979) are inadvisable for the GO, as already pointed out by Moll and Martin (1974), in that these names imply properties which have not been demonstrated for the GO. The use of these designations in the case of greening is probably responsible for the unnecessary confusion created by Nienhaus and Sikora (1979) when they stated that the structures "observed in the phloem tissue of trees affected with citrus greening . . . were also found in the vascular fluid of citrus trees affected with young tree decline . . . in Florida"! On the basis of available evidence, the GO's in the phloem of greening-affected citrus trees and the organisms recovered from the xylem of young tree decline-affected citrus trees, and healthy trees (Feldman and Hanks, 1980) are morphologically different and seem entirely unrelated.

In spite of the arguments that have been developed since 1970 against the mycoplasma-like nature of the GO, the term MLO is still used for it (Su and Chang, 1976; Tanaka and Doi, 1976; Chen, 1979; Iida, 1979; Nariani *et al.*, 1980; S. Attathom *et al.*, personal communication). We hope that the new evidence of the gracilicute nature of the GO will eliminate the use of the erroneous term MLO for GO.

Finally, Indian workers have claimed to have cultured the greening "mycoplasma" on artificial PPLO agar medium (Ghosh *et al.*, 1971) and proved Koch's postulates (Raychaudhuri *et al.*, 1974; Nariani *et al.*, 1972). According to these results the greening pathogen would be a cultivable mycoplasma: our data do not agree with this conclusion.

ACKNOWLEDGMENT

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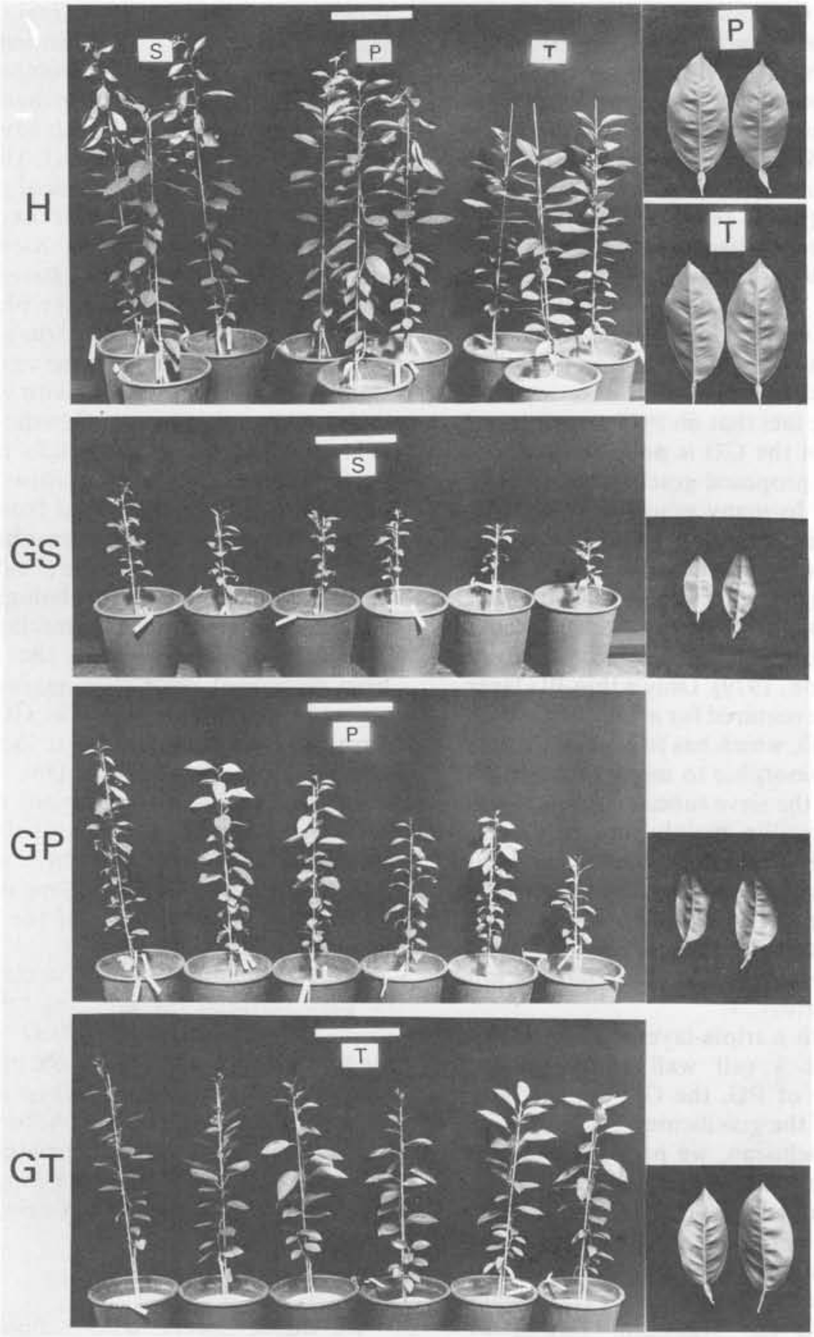

Fig. 3. Effect of penicillin and tetracycline treatments on growth of healthy and greening-affected sweet orange budlings.

H. Healthy Madam Vinous sweet orange on Troyer citrange (back row) or sour orange (front row) treated with nutrient solution alone (S) or containing penicillin (P) or tetracycline (T). Leaves of penicillin (P) and tetracycline (T) treated plants are shown at right.

GS, GP, GT. Greening-affected Hamlin sweet orange on Troyer citrange, treated with nutrient solution alone (S) or containing penicillin (P) or tetracycline (T). No difference was noted between Indian greening-, leaf mottling-, or likubin-affected plants whatever the treatment. Leaves from treated plants are at right.

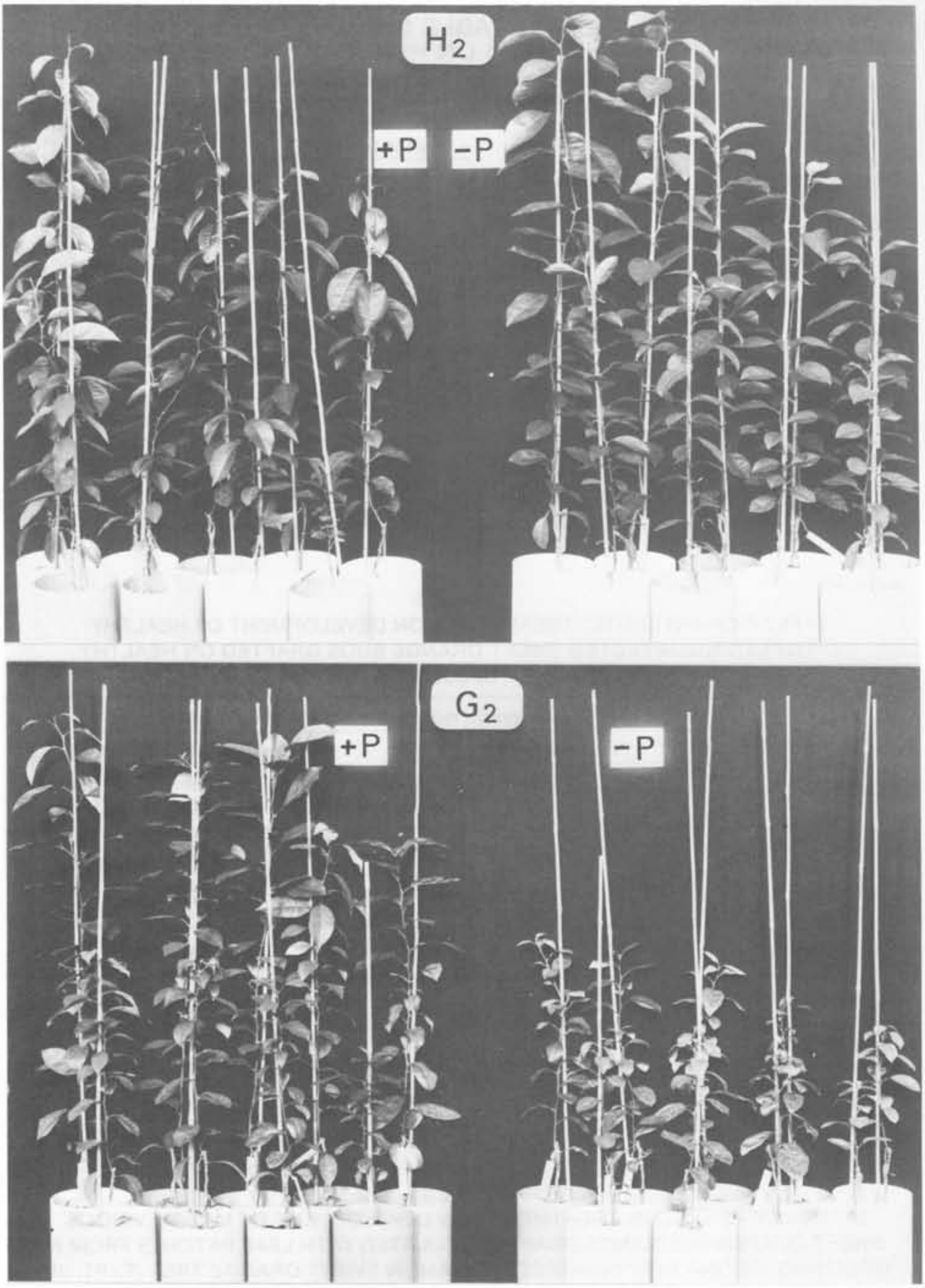

Fig. 4. Healthy (H2) and Indian greening-infected (G2) Madam Vinous sweet orange seedlings 7 months after graft inoculation. Plants received nutrient without (-P) or with (+P) penicillin.

TABLE 1
EFFECT OF PENICILLIN (P) ON HEALTHY AND GREENING-AFFECTED SWEET ORANGE SEEDLINGS (EXPT. 1)

Plant Material	Weeks after treatment	Number of new leaves per plant* -P	Number of new leaves per plant* +P	Length (cm) of new shoots per plant* -P	Length (cm) of new shoots per plant* +P
Healthy					
	11	46	42	93	99
	18	66	78	193	220
Greening-affected					
	11	3	32	29	60
	18	9	43	41	82

* Mean values, 3 replications for healthy, 10 replications for greening (combined values from 4 isolates).

TABLE 2
EFFECT OF ANTIBIOTIC TREATMENTS ON DEVELOPMENT OF HEALTHY OR GREENING-AFFECTED SWEET ORANGE BUDS GRAFTED ON HEALTHY ROOTSTOCK SEEDLINGS (EXPT. II)

Time after budding	Average length (cm) of shoots* from: Healthy buds† No tmt.	Healthy buds† Peni-cillin	Healthy buds† Tetra-cycline	Greening-affected buds‡ No tmt.	Greening-affected buds‡ Peni-cillin	Greening-affected buds‡ Tetra-cycline
33 weeks	56	41	28	20	28	29
55 weeks	148	84	45	51	73	47
21 months§	101	106	66	27	57	62

* Plants were trained to a single shoot.
† Average from 5 shoots.
‡ Average from 15 shoots.
§ Shoots were cut down to 2 buds above bud union 56 weeks after budding (August 31, 1978), and one bud was allowed to grow out.

TABLE 3
EFFECT OF PENICILLIN TREATMENTS ON DEVELOPMENT OF MADAM VINOUS SWEET ORANGE SEEDLINGS GRAFT-INOCULATED WITH LEAF PATCHES FROM A GREENING (POONA STRAIN)-AFFECTED HAMLIN SWEET ORANGE TREE (EXPT. III)

Time after inoculation	Average length (cm) of shoots* from Healthy seedlings -P	Healthy seedlings +P	Greening-affected seedlings -P	Greening-affected seedlings +P
7 months	73	67	39	66
9 months	81	81	47	70

* Average of 9 seedlings.

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