EFFECTS OF TETRACYCLINE ANTIBIOTICS ON PLANTS AFFECTED BY LEGUME LITTLE LEAF DISEASE

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Abstract

* Nicotiana glutinosa, aster, and tomato plants affected by legume little leaf disease were treated by spraying the foliage with 100 μg/ml aqueous solutions of either Achromycin (tetracycline hydrochloride) or Aureomycin (chlorotetracycline hydrochloride), every 2–3 days for periods of 4–8 weeks. Disease symptoms were suppressed in the new growth of all plants during the treatment period, but reappeared 2–4 weeks after the last application of antibiotic. Mycoplasma-like bodies were abundant in the phloem sieve tubes of untreated aster, and in aster which redeveloped symptoms after the antibiotic treatment had been discontinued. In contrast, Mycoplasma-like bodies could not be detected by thin-section electron microscopy in symptomless shoots of Achromycin-treated plants immediately after a period of treatment. In concomitant leafhopper transmission tests with the vector Orosius argentatus, the little leaf agent was not recovered from the symptomless treated shoots, but was readily recovered from diseased shoots which developed after termination of the Achromycin treatment.

Symptomless shoots were detached from tomato plants after a period of tetracycline treatment and were either grafted to Datura stramonium indicator plants or established as rooted cuttings. Little leaf symptoms appeared in both groups of plants, showing that the originally symptomless tomato shoots were infected with the little leaf agent. In thin-section electron microscopy of the symptomless treated tomato shoots, typical Mycoplasma-like bodies were absent, although a few atypical bodies were observed in phloem sieve tubes.

The correlation of tetracycline-induced symptom suppression with apparent absence of Mycoplasma-like bodies from new symptomless shoots and with the failure of leafhoppers to acquire the little leaf agent from these shoots is interpreted as supporting evidence for the hypothesis that legume little leaf disease is caused by a Mycoplasma-like organism.

I. Introduction

Electron-microscopic studies of plants affected by legume little leaf disease have revealed Mycoplasma-like bodies in the sieve tubes of the phloem tissue (Bowyer et al. 1969; Bowyer and Atherton 1970). Similar bodies have been found in the salivary glands and alimentary canals of infective individuals of Orosius argentatus Evans, the leafhopper vector of the little leaf disease, and also in little leaf-infective dodder (Cuscuta australis R. Br.) (Bowyer and Atherton 1971). Mycoplasma-like

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bodies have never been observed in healthy plants, in non-infective leafhoppers, or in non-infective dodder. These results suggest that the little leaf disease is of mycoplasmal, rather than viral aetiiology. However, attempts to culture the agent in vitro have so far been unsuccessful.

Further evidence supporting the hypothesis of mycoplasmal aetiology would be provided by the therapeutic effect of substances known to be active against Mycoplasma-type organisms. As a group, the mycoplasmas are inhibited by tetracycline compounds (Newnham and Chu 1965; Arai et al. 1967), but not by penicillin [except Mycoplasma neurolyticum, which Wright (1967) reported to be penicillin-sensitive]. Ishiie et al. (1967) showed that treatment of yellows-diseased plants with tetracyclines resulted in the suppression of disease symptoms. Doi et al. (1967) reported that Mycoplasma-like bodies, which were abundant in untreated control plants, could not be found by electron microscopy of treated plants. Use of tetracyclines or chloramphenicol to suppress symptoms in yellows-diseased plants, and to reduce or prevent transmission by leafhopper vectors, has since been reported for some of the other diseases with which Mycoplasma-like bodies have been associated (Davis, Whitcomb, and Steere 1968; Brčak et al. 1969; Cousin and Staron 1969; Freitag and Smith 1969; Granados 1969; Hull, Horne, and Nayar 1969; Story and Halliwell 1969; Davis and Whitcomb 1970; Whitcomb and Davis 1970).

This paper reports (1) suppression of symptoms in new growth of little leaf-diseased plants by Achromycin (tetracycline hydrochloride) and Aureomycin (chlortetracycline hydrochloride); (2) correlation of symptom suppression with apparent absence of Mycoplasma-like bodies from the new symptomless shoots, and with failure of leafhoppers to acquire the little leaf agent from these shoots; (3) persistence of little leaf infectivity in symptomless shoots removed from diseased plants after a period of Achromycin treatment.

II. Materials and General Methods

The strain of the legume little leaf agent was the same as that used previously (Bowyer and Atherton 1970), and was maintained in Datura stramonium L., inoculated by the vector O. argentinensis. Lettuce necrotic yellows virus was obtained from commercially grown lettuce, near Brisbane, and when mechanically inoculated into Nicotiana glutinosa L. seedlings, produced symptoms similar to those described by Stubbs and Grogan (1963). All experiments were carried out in an insect-proof glasshouse.

Three species of little leaf-diseased plants were treated with either Achromycin or Aureomycin by spraying the foliage, after symptom development, with a 100 μg/ml aqueous solution of the antibiotic. The species used were aster [Callistephus chinensis (L.) Nees.], N. glutinosa, and tomato (Lycopersicon esculentum Mill.). Aster and N. glutinosa were infected by leafhopper inoculation of seedling plants; tomato seedlings were infected by eleft-grafting them with diseased tomato seions. Symptoms of little leaf disease were well advanced in all plants before commencement of antibiotic treatment. Symptoms had been present for approximately 14 days, and included severe interveinal chlorosis, axillary shoot proliferation with small leaves, and the green flower ("viressence") syndrome. Lettuce necrotic yellows virus symptoms in N. glutinosa consisted of severe stunting, leaf distortion, and chlorotic leaf lesions. Plants were sprayed to "dripping point" every second or third day with the appropriate antibiotic solution or distilled water, on the upper surfaces of the leaves.

Procedures for thin-section electron microscopy were described previously (Bowyer and Atherton 1970).
CHEMOTHERAPY OF LEGUME LITTLE LEAF DISEASE

III. EXPERIMENTAL AND RESULTS

(a) Effects of Antibiotics on Disease Symptoms

Equal numbers of little leaf-diseased plants were sprayed with tetracyclines and with distilled water as controls. Further controls consisted of the following: (1) little leaf-diseased \textit{N. glutinosa} plants sprayed with 100 \(\mu\)g/ml aqueous penicillin G solution; (2) \textit{N. glutinosa} plants infected with lettuce necrotic yellows virus, half of which were sprayed with 100 \(\mu\)g/ml aqueous Achromycin, and the other half with distilled water.

\textbf{Table 1}

\textbf{Response by Plants Affected by Little Leaf (LL) and Lettuce Necrotic Yellows Virus (LNYV) to Treatment with Tetracyclines or Penicillin-G}

Plants were treated by spraying the foliage with 100 \(\mu\)g/ml aqueous solution of antibiotic, beginning 14 days after symptom development; control plants were sprayed with distilled water.

<table>
<thead>
<tr>
<th>Antibiotic</th>
<th>Species treated</th>
<th>Disease</th>
<th>No. of applications/period of treatment (days)</th>
<th>No. of plants showing recovery/total No. treated</th>
</tr>
</thead>
<tbody>
<tr>
<td>Achromycin</td>
<td>\textit{N. glutinosa}</td>
<td>LL</td>
<td>10/28</td>
<td>8/8</td>
</tr>
<tr>
<td></td>
<td>\textit{N. glutinosa}</td>
<td>LNYV</td>
<td>10/28</td>
<td>0/4</td>
</tr>
<tr>
<td>Aster</td>
<td></td>
<td>LL</td>
<td>17/49</td>
<td>10/10</td>
</tr>
<tr>
<td>Tomato</td>
<td></td>
<td>LL</td>
<td>28/55</td>
<td>3/3</td>
</tr>
<tr>
<td>Aureomycin</td>
<td>\textit{N. glutinosa}</td>
<td>LL</td>
<td>10/28</td>
<td>4/4</td>
</tr>
<tr>
<td>Penicillin G</td>
<td>\textit{N. glutinosa}</td>
<td>LL</td>
<td>10/28</td>
<td>0/4</td>
</tr>
</tbody>
</table>

The results of the experiment are summarized in Table 1. All little leaf-diseased plants treated with tetracyclines showed a very marked response in terms of suppression of disease symptoms in the new growth which developed during the treatment. In \textit{N. glutinosa} treated with either Achromycin or Aureomycin, recovery was apparent after only two applications of antibiotic. The most obvious effect was the almost immediate suppression of axillary shoot proliferation. Apical dominance was restored, and leaves of the new growth developed to normal size, free of the characteristic "vein clearing" and interveinal chlorosis present in the leaves of untreated plants. The leaves of proliferating axillary shoots near the bases of the plants before commencement of treatment also developed to relatively normal size. The only apparent phytotoxic effect of the tetracyclines was slight chlorosis of the youngest leaves, but this disappeared as the leaves matured. Figure 1 shows representative plants of \textit{N. glutinosa} before and after Aureomycin treatment.

Achromycin treatment of tomato and aster also resulted in suppression of little leaf symptoms, although the response was slower than in \textit{N. glutinosa}. In aster, recovery was apparent after 2–3 weeks. The most prominent feature was the development of small, pink flowers on plants which, before treatment, were producing green flowers. Treated tomato plants also produced symptomless vegetative growth and
normal flowers, from which normal fruit developed. Thirty-three of 40 seeds collected from one such fruit germinated and produced healthy plants. Untreated plants produced green, sterile flowers.

None of the lettuce necrotic yellows virus-infected *N. glutinosa* plants treated with Achromycin showed recovery from the disease. Penicillin had no observable effect on little leaf symptoms in *N. glutinosa*.

![Image of lettuce plants showing symptoms and treatment results](image)

**Fig. 1.**—Aureomycin suppression of little leaf disease symptoms in *N. glutinosa*. Inset: plant on right before commencement of treatment, showing typical early symptoms of little leaf disease, including green flowers (at apex) and extensive shoot proliferation; right: same plant after spraying 10 times at 3-day intervals with 100 μg/ml Aureomycin—note the new symptomless growth and suppression of symptoms in the original basal axillary shoots; left: untreated control plant, showing advanced little leaf symptoms—severe interveinal chlorosis of older leaves and extreme proliferation of axillary shoots with very small, puckered leaves.

(b) Tests on Tetracycline-treated Plants

(i) *Aster*

(1) *Electron Microscopy.*—At the end of the 7-week chemotherapy period, samples from two Achromycin-treated and two control plants were prepared for thin-sectioning. Samples from the two treated plants consisted of stem segments of new symptomless shoots which developed during the treatment. Samples from the
two control plants were of similar age to those from the treated plants, and consisted of stem segments of small proliferating shoots near the apices of the plants. No Mycoplasma-like bodies were observed in a total of 50 sections of the antibiotic-treated specimens. In contrast, large numbers of Mycoplasma-like bodies were present in the phloem sieve tubes in all 10 sections examined from the untreated control material.

(2) Leafhopper Assay.—At the time of preparing specimens for electron microscopy, symptomless shoots on the two Achromycin-treated plants were tested for little leaf infectivity using O. argentatus as a means of assay. To prevent access of leafhoppers to older infected tissues, all old leaves were removed, and the stems developed before treatment were wrapped with several layers of aluminium foil. The test leafhoppers thus had access only to symptomless new shoots developed during the treatment. Fifth-instar nymphs were given a 3-day acquisition feed, and then transferred to healthy D. stramonium for 21 days to allow for completion of the incubation period. Forty leafhoppers from each of the two plants were then transferred to fresh groups of five D. stramonium test seedlings.

None of the 10 D. stramonium test plants developed little leaf symptoms, indicating that the test leafhoppers did not acquire the little leaf agent during the 3-day access to symptomless shoots which developed in response to Achromycin treatment. In contrast, 9 of 10 D. stramonium plants used to test the infectivity of leafhoppers fed for 3 days on two untreated (control) aster plants developed typical little leaf symptoms 19–24 days after exposure to the test leafhoppers.

(3) Tests following Recurrence of Symptoms.—Little leaf symptoms reappeared in the two treated plants 15 and 20 days respectively after the last application of Achromycin. Young shoots showing typical symptoms were then prepared for electron microscopy, which revealed large numbers of typical Mycoplasma-like bodies in the phloem sieve tubes. At the same time, non-infective leafhoppers were fed on the plants for 3 days, and 40 from each plant were tested on groups of five D. stramonium seedlings. All 10 test plants developed little leaf symptoms, showing that the little leaf agent was readily recovered by leafhoppers from plants in which symptoms recurred following a tetracycline-induced remission period.

(ii) Tomato

(1) Shoots Grafted to Indicator Plants.—Eight weeks after commencing tetracycline therapy, five symptomless axillary shoots from one of the treated plants were cleft-grafted to D. stramonium seedlings. After grafting, neither the tomato scions nor the D. stramonium stock plants received further antibiotic. As controls, five scions from one of the untreated diseased tomato plants were grafted to D. stramonium seedlings. All grafted plants were then maintained under similar conditions in the glasshouse.

Little leaf symptoms appeared in the symptomless tomato shoots used as scions 25–29 days after grafting, i.e. after termination of antibiotic treatment of the source plant. Symptoms developed in the D. stramonium indicator plants 45–55 (mean 49·6) days after grafting. In contrast, symptom appearance in the D. stramonium plants grafted with scions from the untreated diseased tomato plant occurred
23–31 (mean 27.8) days after grafting. These results show that although Achromycin treatment significantly delayed symptom development in the *D. stramonium* test plants by 2–4 weeks, the symptomless treated tomato scions were still infected with the little leaf agent.

After symptom development in the *D. stramonium* indicator plants grafted with scions from the Achromycin-treated tomato, the presence of *Mycoplasma*-like bodies in two of these test plants was confirmed by thin-section electron microscopy of petioles bearing typically small leaves.

(2) **Shoots Established as Rooted Cuttings.**—From a little leaf-diseased tomato plant which had received 28 applications of Achromycin over a period of 8 weeks, four symptomless shoots which developed during the treatment were removed and placed in water for several days. The cuttings developed adventitious roots, and were then transferred to pots of soil. Two of the rooted cuttings were sprayed 10 times with 100 μg/ml Achromycin at 2-day intervals, and the other two served as untreated controls.

None of the four plants remained free of little leaf symptoms. The two plants which did not receive antibiotic after propagation developed symptoms after 18 and 26 days respectively. The two plants sprayed with Achromycin for 20 days after propagation remained healthy during this time, but symptoms appeared 22 and 25 days respectively after the last treatment.

(3) **Electron Microscopy of Symptomless Shoots.**—Segments of leaf petioles from the shoots of an Achromycin-treated and a control tomato plant were prepared for thin sectioning when the shoots were grafted to the *D. stramonium* indicator plants in (1). A total of approximately 100 sections of the petioles of two shoots from the treated plant were examined, but no typical *Mycoplasma*-like bodies were observed. However, a total of seven sieve tube elements contained small numbers (10–20) of pleomorphic, membrane-bound structures approximately 200–600 nm in diameter. The limiting membranes were poorly defined, and the only apparent internal structures were densely staining ribosome-like particles. The identity of these membrane-bound structures is uncertain, but it seems possible that they were *Mycoplasma*-like bodies, the ultrastructure of which had been affected by the tetracycline treatment of the host plant. In 20 sections of the control material, most of the sieve tubes contained large numbers of typical *Mycoplasma*-like bodies, which often occupied the entire lumen of a cell.

**IV. Discussion**

The suppression of symptoms in the new growth of plants treated with Achromycin or Aureomycin, and the lack of response to penicillin, support the direct electron-microscopic evidence for mycoplasmal aetiology of the little leaf disease (Bowyer and Atherton 1970, 1971). This is strengthened by the failure of Achromycin to suppress the known symptoms caused by lettuce necrotic yellows virus. Furthermore, electron-microscopic examination of symptomless shoots of aster, which developed in response to tetracycline treatment, indicated that *Mycoplasma*-like bodies were either absent or so reduced in numbers that they were not detected by this technique. This result is consistent with the failure of *O. argentatus* to acquire
the little leaf agent from the symptomless shoots during a 3-day feed following the last Achromycin treatment. Failure to observe *Mycoplasma*-like bodies by electron microscopy of symptomless shoots of mulberry following tetracycline therapy of diseased plants was reported by Doi et al. (1967). Other workers have reported reduced leafhopper recovery of the aster yellows agent from tetracycline-treated plants (Freitag and Smith 1969; Davis and Whitcomb 1970). The present work has established that tetracycline-induced suppression of little leaf symptoms is correlated with (1) apparent absence of *Mycoplasma*-like bodies from new symptomless shoots as indicated by electron microscopy, and (2) failure of leafhoppers to acquire the little leaf agent from these shoots. Furthermore, the recurrence of symptoms following the termination of tetracycline therapy was correlated with the presence of large numbers of typical *Mycoplasma*-like bodies in the new diseased growth, and the concomitant recovery of the little leaf agent from the plants by leafhoppers. These results help further to implicate the *Mycoplasma*-like bodies in the aetiology of the little leaf disease.

Symptom recurrence following the tetracycline-induced remission period was observed in all plants 2–4 weeks after the last application of antibiotic. This result suggests that the antibiotics inhibited the multiplication of the little leaf agent, and it may parallel the observation that mycoplasmal contamination of animal cell cultures is generally suppressed, but not eliminated, by the inclusion of antibiotics in the culture medium (Cross, Goodman, and Shaw 1967; Schweizer, Witzleb, and Blumohr 1970). It is clear that foliar application of these antibiotics allows the survival of some *Mycoplasma*-like organisms in at least some cells of the treated plants. The 2–4 week period between the last application of antibiotic and the reappearance of symptoms might represent an incubation period required by the surviving organisms to multiply and systemically reinfect the plant. It is probably significant that this period is similar to the original incubation period between leafhopper or graft inoculation of plants and the appearance of symptoms.

The results of foliar application of tetracyclines to plants affected by other yellows diseases also indicated that the antibiotics have only a temporarily suppressive effect on the disease agents (Ishiie et al. 1967; Davis, Whitcomb, and Steere 1968; Cousin and Staron 1969). However, Granados (1969) found that immersion of plant roots in 1000 µg/ml Achromycein or Aureomycin, immediately after the plants were inoculated with the corn stunt agent, prevented symptom development in some plants. Shikata, Teng, and Matsumoto (1969) reported that immersion of white leaf-diseased sugar-cane sets in tetracycline solutions apparently prevented symptom development in the resulting plants. Thus it appears that under certain conditions, dependent upon factors such as antibiotic concentration and mode of application, tetracycline treatment of yellows-diseased plants eliminates the disease agents.

Previous investigations apparently have not involved the study of symptomless shoots detached from yellows-diseased plants after a period of tetracycline therapy. It was thought that the completely symptomless shoots developing wholly within the chemotherapy period might be free of the disease agent if detached from the parent plant immediately after the last application of antibiotic. It is conceivable that the roots and original infected stems of plants treated by foliar spraying might act as reservoirs of the little leaf agent which survived the treatment and subsequently
caused reinfection. However, the presence of the little leaf agent in symptomless shoots was shown firstly by the appearance of symptoms in the plants resulting from vegetative propagation of symptomless tomato shoots, and secondly by symptom development in tomato shoots grafted to D. stramonium indicator plants, and later in the D. stramonium test plants themselves. As expected, electron-microscopic examination of these plants revealed numerous Mycoplasma-like bodies in the phloem sieve tubes. This established that the bodies were translocated to the new tissue developing during treatment, although electron-microscopic examination of such tissue indicated that typical Mycoplasma-like bodies could not be detected. Whether the small numbers of unidentified membrane-bound structures observed in the sieve tubes of new symptomless shoots were atypical mycoplasmas affected by tetracycline treatment, will have to be determined by further work.

Although the results of the present work are consistent with the hypothesis of a mycoplasmal aetiology of little leaf disease, they do not exclude the possibility that the Mycoplasma-like organisms present in diseased plants are not the primary disease agents. As Davis and Whitecomb (1970) have pointed out in their work with aster yellows, the organisms may be secondary pathogens, and the therapeutic effectiveness of antibiotics may be due to modification of host susceptibility to some unknown primary disease agent. However, there is at present no evidence for such an agent, but proof of mycoplasmal aetiology of little leaf and other yellows diseases will require cultivation of the organisms in vitro, and satisfaction of Koch’s postulates.

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VI. References


