# Growth of *Phytophthora citrophthora* and *Deuterophoma tracheiphila* on Culture Media Containing Leaf Extracts of Healthy and Virus-Infected Plants

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THE FACT THAT bean plants infected with tobacco mosaic virus showed resistance to rust was reported by E. M. Wilson (14). King *et al.* (7) observed that red clover plants, infected with bean yellow mosaic virus, are resistant to *Erysiphe polygoni*. R. D. Watson and J. M. Guthrie (13) reported that virus infections increased the susceptibility of red clover to infection by root-rot fungi. G. N. Agrios (1) reported that *Cytospora* sp., *Sphaeropsis malorum*, and *Phoma* sp. grew better on extracts of fruit and leaves from apple and pear trees infected with certain virus diseases.

In Brazil, Rossetti *et al.* (10) found that when the cambial zone of citrus trees severely affected with tristeza or exocortis viruses was inoculated with *Phytophthora citrophthora*, the fungus grew very slowly in comparison with its growth on vigorous trees. Also, *P. citrophthora* grew poorly on the stems of exocortis-infected Etrog citron 60-13 (*Citrus medica* L.) plants in the greenhouse as compared with its growth on virus-free plants.

Systemic virus diseases may induce changes in the substances present

in the host plant that are essential for growth of the fungus. For example, Diener and Dekker (4) found L-pipecolic acid in leaves of peach trees infected with western-X disease, and later Diener (5) found that the amino acid content in virus-infected cherry and peach leaves increased. Rossetti and Bitancourt (9) found that lysine increased and arginine decreased in the bark of exocortis-infected citrus trees as compared with healthy ones. A. W. Feldman and R. W. Hanks (6) reported that the amino acid content of leaves of exocortis-infected sweet orange trees was less than that of healthy ones.

This paper presents the results of investigations, of fungi pathogenic to citrus species, made for the purpose of detecting changes in substances produced by the host plants in response to virus infection.

## *Materials and Methods*

Leaves were collected from actively growing twigs of 1-year-old greenhouse plants of Etrog citron 60-13 infected with exocortis and leaf curl viruses, 1-year-old Eureka lemon plants infected with infectious-variegation and crinkly-leaf viruses, and Troyer citrange plants infected with tatter-leaf virus. Leaves of virus-free plants of the same varieties of the same age and grown under the same conditions were also collected. After collections, the leaves were thoroughly washed.

The exocortis virus was a severe strain sent by E. C. Calavan. The viruses, tatter leaf, and crinkly-leaf strain 421-A-65, were obtained from J. M. Wallace, California. Infectious-variegation strain 1234 was secured from T. J. Grant, Florida, and the leaf-curl virus was obtained from A. A. Salibe, Limeira, Brazil. This last strain also contained tristeza and possibly psorosis viruses.

Two experiments were performed. In the first, 20 g of leaves were ground with 60 ml of water in a Virtis 45 at full speed for 2 min. Ten grams of healthy and of virus-infected leaves of Troyer citrange plants were similarly ground with 30 ml of water. The juice obtained from each sample was strained through cheesecloth and centrifuged in a refrigerated centrifuge at 10,000 g for 20 min.

Nutrient media were prepared as follows: each contained 50 ml of the supernatant liquid from the Etrog citron or from the Eureka lemon material; in the case of Troyer citrange material, 30 ml of supernatant liquid was used; to these amounts of the leaf extract, 50 ml of double distilled water, 1.5 g of agar, and 1 ml of Paul Smith (12) major and minor elements solution were added. The pH of the leaf extract media varied from 6.5 to 6.8. All media were autoclaved at 120°C for 20 min.

The amount of 4.5 ml was poured into 5 cm petri dishes and left to solidify.

Pure cultures of *P. citrophthora* and *Deuterophoma tracheiphila* were used. The *P. citrophthora* culture, 147E, from the Instituto Biologico of São Paulo, Brazil, was isolated from a naturally infected sweet orange tree in Argentina by Bitancourt and Fawcett in 1939. Since then, it has been maintained on potato dextrose agar and is revived from time to time by inoculation on citrus plants and reisolation. The culture of *D. tracheiphila* was provided by the Plant Pathology Institute of Bologna, Italy.

Inoculum of both cultures consisted of 2 mm disks of agar carrying the fungus, taken from the periphery of colonies growing on water agar. The inoculum was placed in the center of 10 dishes for each treatment. All treatments were grown under the same conditions. Every 24 hours during a period of 5 days, the radial growth of the fungi was measured as the mean of 2 diameters of each colony.

In the second experiment, extracts of exocortis-infected and healthy Etrog citron plants, and of infectious-variegation-infected, crinkly-leaf-infected, and healthy Eureka plants were prepared following the method described. For each treatment, 60 g of leaves were ground with 180 ml of water. Nutrient media, containing 150 ml of the leaf extract, 150 ml of double distilled water, and 3 cc of Paul Smith major and minor elements solution, were prepared and solidified with 4.5 g of Difco agar. After autoclaving, 15 ml was poured into 9 cm diameter petri dishes and left to solidify. The surface of the solidified medium in some of the petri dishes was covered with a 9 cm diameter disk of sterilized cellophane paper.

In this experiment, only *P. citrophthora* was used. For each treatment, the fungus inoculum was placed in the centers of 20 petri dishes, directly over the culture media on 10 of them and over the cellophane paper on the other 10. The dishes were kept at 24°C. After 6 days radial growth of the cultures was measured as described previously. The mycelial mat was then removed from the cellophane disk, dried at 75°C for 6 hr, and weighed. The cultures were also examined under the microscope, and microphotographs were taken. The fungus growth data were analyzed statistically by the method of analysis of variance, and the Duncan test of significance was used.

In both experiments the fungus inoculum was also placed on plain water agar medium without any leaf extract. The fungus was allowed to grow under the same conditions as on the leaf extract media. Growth on

this medium was very scant and measurements were not considered.

In both experiments an aliquot of the supernatant liquid resulting from centrifuging the leaf extract was kept for chemical analysis. The dry weight of 10 ml of the supernatant liquid was determined after drying 15 hr at 90°C. Proteins in the supernatant were determined according to the method of Lowry *et al.* (8). The elements N, P, K, Ca, and Mg were determined by standard methods.

## *Results*

FUNGUS GROWTH ON MEDIA PREPARED WITH LEAF EXTRACT FROM EXOCORTIS-INFECTED, LEAF CURL-INFECTED, AND EXOCORTIS-FREE ETROG CITRON PLANTS.—In the first experiment (Table 1), *P. citrophthora* grew 39.3 per cent better and *D. tracheiphila* grew 9.6 per cent better on extract medium prepared from exocortis-infected leaves than on medium from virus-free leaves (control). The two fungi grew 5 per cent and 2.2 per cent better, respectively, on leaf curl-infected leaf extract medium than on the virus-free leaf extract medium. The growth of both *P. citrophthora* and *D. tracheiphila* was significantly better on exocortis-infected leaf extract medium than on the leaf-curl extract medium or on the control.

In the second experiment (Table 1), where the media was not covered with cellophane, radial growth of *P. citrophthora* was 10.7 per cent better on the exocortis-infected leaf extract medium than on control medium prepared from virus-free leaf extract. The growth increase was statistically significant, which confirmed the results obtained in the first experiment. There was no difference in radial growth in the dishes with cellophane, but the dry weight of the mycelial mat was significantly higher (31.8 per cent) on the media from exocortis-infected material than on the control media. In the first case, the hyphae were much more intensely ramified as compared with hyphae growing on the control medium. Also, in the first case the fungus grew completely superficially with no digestion of the cellophane, whereas on the control media some digestion of cellophane occurred which allowed the fungus to grow, although very poorly, on the medium underneath it.

FUNGUS GROWTH ON INFECTIOUS-VARIEGATION-INFECTED, CRINKLY-LEAF-INFECTED, AND VIRUS-FREE EUREKA LEMON LEAF EXTRACT MEDIA.—In the first experiment, *P. citrophthora* grew significantly better on agar media prepared from extract from crinkly-leaf-infected leaves, and slightly, but not significantly, better on extract from infectious-variegation-infected leaves than on agar containing extract from virus-free leaves (control).

TABLE 1. GROWTH OF *Phytophthora citrophthora* AND *Deuterophoma tracheiphila* IN CULTURE MEDIA CONTAINING LEAF EXTRACTS OF HEALTHY AND VIRUS-INFECTED CITRUS PLANTS

| Variety | Virus content | First experiment: Mean diameter in cm of *Phytophthora* | First experiment: Mean diameter in cm of *Deuterophoma* | Second experiment: Mean diameter of *Phytophthora* cultures in cm, no cellophane | Second experiment: Mean diameter of *Phytophthora* cultures in cm, with cellophane | Second experiment: Dry weight of *Phytophthora mycelium*, mg |
|---|---|---|---|---|---|---|
| Etrog citron (60-13) | leaf-curl | 3.39 | 3.73 | | | |
| | exocortis | 4.50[a] | 4.00[b] | 7.450[a] | 8.295 | 44.76[a] |
| | virus-free (control) | 3.23 | 3.65 | 6.655 | 8.338 | 33.95 |
| Eureka lemon | infectious variegation | 3.13 | 3.96[a] | 6.390 | 7.575[a] | 33.20[a] |
| | crinkly leaf | 3.33[a] | 3.80 | 7.395[a] | 8.735 | 36.70[a] |
| | virus-free (control) | 2.99 | 3.76 | 6.875 | 8.670 | 22.15 |
| Troyer citrange | tatter leaf | 4.21 | 4.41[a] | | | |
| | virus-free (control) | 4.28 | 3.83 | | | |

a. Significant at the 1.0 per cent level (Duncan test).
b. Significant at the 5 per cent level.

TABLE 2. DRY WEIGHT, PROTEIN CONTENT, AND MINERAL SALTS OF THE LEAF EXTRACTS OF HEALTHY AND VIRUS-INFECTED CITRUS PLANTS, IN MILLIGRAMS PER MILLILITER

| Variety | Virus content | Dry weight | Protein | N, total | P | K | Ca | Mg |
|---|---|---|---|---|---|---|---|---|
| Etrog 60-13 | exocortis | 28.12 | 11.3 | 1.709 | 0.145 | 0.915 | 0.810 | 0.158 |
| | virus-free (control) | 20.19 | 7.3 | 1.044 | 0.139 | 0.827 | 0.148 | 0.058 |
| Eureka | crinkly leaf | 27.85 | 14.3 | 1.425 | 0.250 | 1.070 | 0.202 | 0.100 |
| | infectious variegation | 21.12 | 10.0 | 1.002 | 0.102 | 0.401 | 0.126 | 0.046 |
| | virus-free (control) | 21.40 | 10.3 | 0.880 | 0.079 | 0.728 | 0.217 | 0.078 |
| Troyer citrange | tatter leaf | 33.86 | 19.5 | 1.756 | 0.155 | 1.505 | 0.328 | 0.110 |
| | virus-free (control) | 27.07 | 15.5 | 1.675 | 0.165 | 0.800 | 0.183 | 0.104 |

The radial growth increase over the control was 11.4 per cent in the first case and 4.7 per cent in the second case.

The growth of *D. tracheiphila* on agar containing extract from infectious-variegation-infected leaves was 5.4 per cent better than on the control medium prepared from virus-free leaf extract and was significantly better than its growth on extract of crinkly-leaf-infected leaves or on the control medium.

In the second experiment, radial growth of *P. citrophthora* was 7 per cent better on the crinkly-leaf-infected leaf extract medium than on the control medium, or on the medium not covered with cellophane. The growth increase was significant and confirmed results obtained in the first experiment on media without cellophane. On the same medium cov-

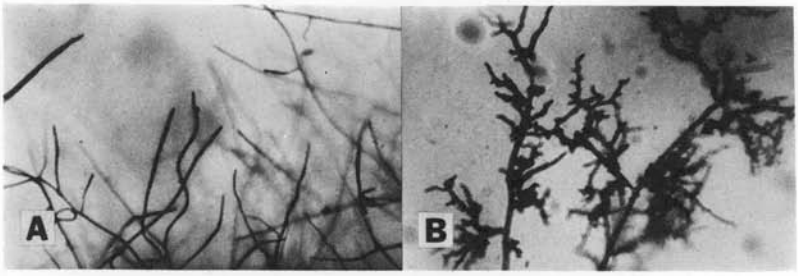


FIGURE 1. *Hyphae of* P. citropthora *growing on: A. culture medium containing extract of healthy Eureka lemon leaves; B. culture medium containing extract of crinkly-leaf-infected Eureka lemon.*

ered with cellophane, there were no differences in radial growth. As in the first experiment on the medium with infectious-variegation-infected leaf extract, there was no growth increase as compared with the control. On the same medium covered by cellophane, radial growth was significantly lower (12.6 per cent) than on the control.

The dry weight of the mycelial mat growing on infectious-variegation media and on crinkly-leaf-infected leaf extract media, respectively, was significantly higher than on control. The increase in dry weight over the control was of 49.9 per cent in the first case and 65.7 per cent in the second case. Ramification of hyphae was very conspicuous on both these media, whereas on the control ramification was scant (Fig. 1).

On these three media, the fungus partially digested the cellophane, and a slight growth of mycelium on the medium under the cellophane disk could be detected. Thus, a small portion of the mycelial mat was lost from the dry weight measurements.

FUNGUS GROWTH ON TATTER-LEAF-INFECTED, AND ON VIRUS-FREE

TROYER CITRANGE LEAF EXTRACT MEDIA.—On agar containing extract from tatter-leaf-infected leaves, the growth of *D. tracheiphila* was 15.1 per cent greater than on the control (significant at the 0.01 level), whereas *P. citrophthora* grew equally well on both media.

CHEMICAL ANALYSIS OF THE LEAF EXTRACTS OF HEALTHY AND VIRUS-INFECTED PLANTS.—Data presented in Table 2 show that dry weight, total N, K, and Mg as well as protein, content were higher on leaf extracts from virus-infected leaves than on those from virus-free plants, except in the case of infectious variegation. Phosphorus was found in greater amount in virus-infected leaves than in virus-free leaves, except in the case of tatter-leaf virus. Calcium was found in greater amounts in exocortis-infected than in virus-free Etrog leaves.

## *Discussion*

The results indicate that virus infections induce changes in the relative concentrations of substances present in citrus leaves. These changes may be responsible for the different rate and type of growth of *P. citrophthora* on infected leaf extracts as compared with healthy leaf extracts.

Nutrition, growth, and morphogenesis of *P. citrophthora* were studied by Bitancourt *et al.* (2, 3, 9) who found that several factors act differently on mycelial growth of *P. citrophthora.* A factor which induces elongation of the hyphae is present in common agar, in the bark of citrus trees, potato broth, et cetera, and was designated as "factor L." Ramification of the hyphae (measured by the amount of light that passes through the cultures growing in solid media) is induced by thiamin which has a synergistic effect with factor L. Total growth (measured by the dry weight of mycelial mats growing in liquid media) is much favored when these two factors act together. The development of ramifications from main hyphae is inhibited when oxygen pressure is somewhat reduced.

In the present case, the better radial growth of both *P. citrophthora* and *D. tracheiphila* on media containing extracts from leaves of plants infected by virus diseases may result from the higher dry weight, the higher protein content, and the greater N, Ca, and Mg content of the infected leaves. These components may influence factor L which induces elongation of the hyphae.

The same differences in radial growth were not observed when a thin layer of cellophane was interposed between the fungus and the medium, except for infectious-variegation leaf extracts, in which radial growth was significantly reduced.

Ramification of the fungus hyphae was consistently greater on virus-infected leaf extracts media than on the control media. The factor responsible for this morphological difference has not been determined, but a greater amount of thiamin in the infected leaves could be responsible.

ACKNOWLEDGMENTS.—The authors are indebted to Dr. E. Laville, Head of the Plant Pathology Laboratory of the Institut Français de Recherches Fruitieres Outre-Mer (IFAC) located at the Faculte des Sciences, Orsay, for laboratory facilities, and to Mr. Jackie Tailler, technician at the IFAC Biochemistry Laboratory, Versailles, for his valuable help with the greenhouse work.

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