production of thymine dimers and the increased killing of *E. coli* by irradiation at −79° and −196°C. This suggests that cyclobutane-type thymine dimers do not play as significant a role in the events leading to the death of irradiated frozen cells as they appear to play at room temperature (*7*). These results provide further evidence that the relative biological importance of a given photoproduct can change markedly, depending upon growth or irradiation conditions (*8*).

The photochemical event that does correlate with viability under the present conditions is the cross-linking of DNA with protein. Freezing produces both a change in the rate of formation and in the yield of DNA cross-linked to protein. The freezing cannot be a simple dose-modifying factor because the final yield of cross-linked DNA is different at the different temperatures. Freezing, however, may alter the configuration or the proximity of the protein and DNA within the cells so that the probability of forming DNA-protein cross-links by irradiation is greatly enhanced, thus leading to the greater lethality observed under these conditions.

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Nobiletin Is Main Fungistat in Tangerines Resistant to Mal Secco

Abstract. *A number of crystalline compounds isolated from peel of tangerines resistant to "mal secco" were characterized and tested for fungistatic activity toward* Deuterophoma tracheiphila. *Nobiletin exhibited strong fungistatic activity, tangeritin was weakly active, and hesperidin slightly stimulated fungal growth. Rough lemon seedlings, inoculated with the pathogenic fungus, rapidly developed the symptoms of mal secco, whereas continuous infusion of the inoculated seedlings with nobiletin solution largely prevented appearance of the disease.*

The "mal secco" disease of citrus varieties, caused by the pathogenic fungus *Deuterophoma tracheiphila*, is widespread throughout the Mediterranean (Israel, Egypt, Cyprus, Turkey, Greece, Italy, Southern France) and Black Sea areas. The economic importance of the disease derives from the fact that it greatly reduces the life expectancy of affected lemon groves (*1*); other citrus varieties such as grapefruit also appear to be susceptible. No means of prevention or cure have yet been suggested.

Research on the relation between natural substances and resistance to disease has been carried out mainly with conifers (*2*). Many bioflavonoid compounds have been isolated from citrus plants (*3*), but their physiologic roles in the mechanism of resistance to disease are obscure. Certain unidentified substances have been shown to inhibit growth of *D. tracheiphila* in vitro (*4*), but, as far as I know, no other results have been published. I have tried to identify the substances present in disease-resistant citrus varieties that inhibit the growth of *D. tracheiphila*.

The sources of the isolated substance were dried leaves, bark, or peel of resistant varieties of tangerine (*Citrus reticulata* Bl.) such as Dancy and Cleopatra tangerines and clementines. Water extracts of these materials inhibited growth of the fungus in vitro. The following description refers to dried peel from Dancy tangerines. Initial experiments were made with water extracts, but subsequently I found that methanol extracts had comparable fungistatic activities. Methanol was preferred for preparative work because it extracts less impurities.

Two hundred grams of finely ground tangerine peel were refluxed with 600 ml of methanol for 4 hours. The mixture was filtered, and the residue was similarly extracted four more times. The combined filtrates were concentrated under partial vacuum to 200 ml and left at room temperature for 48 hours. The white precipitate that formed was filtered off (precipitate 1); the filtrate was concentrated to 50 ml, and 200 ml of distilled water was added. The solution was allowed to stand at room temperature for 48 hours longer. A light-brown precipitate was filtered off (precipitate 2); the aqueous methanolic filtrate was extracted with, first, 250 ml and then 100 ml of ethyl acetate. The combined ethyl acetate extracts were dried over sodium sulfate and filtered. The filtrate was concentrated to 35 ml and left for 24 hours at room temperature. A white precipitate was filtered off (precipitate 3). The filtrate was concentrated nearly to dryness, and the residue was dissolved in 15 ml of hot 95-percent ethanol and filtered.

After at least 48 hours at 5°C, a light-yellow crystalline material was obtained and dried at 105°C under partial vacuum, yielding 0.7 g of a crystalline substance. After repeated treatment with active charcoal and recrystallization from methanol, this substance melted at 137° to 138°C; its ultraviolet-absorption spectrum exhibited maxima at 248, 272, and 333 mμ. The crystals showed a yellow fluorescence. Paper chromatography on Whatman No. 1, with a mixture of *n*-propanol and water (2:1) as the developing solvent, yielded a spot with a bluish-white fluorescence and R_F of 0.92. The substance gave a positive flavone reaction with magnesium powder and concentrated hydrochloric acid in alcoholic solution. The absence of free OH groups was indicated by insolubility of the compound in dilute alkali and by the negative $FeCl_3$ reaction. All these characteristics are identical with those reported for 5,6,7,-8,3′,4′-hexamethoxyflavone, or nobiletin (Fig. 1A; *5*, *6*).

Chromatographic fractionation of the mother liquor from which nobiletin had been crystallized showed the presence of another substance, with a strong-yellow fluorescence, that moved to the front of the paper (R_F, 0.95; *n*-propanol and water at 2:1); it also gave a positive flavone reaction with Mg powder and concentrated HCl in

Table 1. Average (five replicates) inhibition of *D. tracheiphila* in vitro by treatment with four concentrations of nobiletin during 12-day incubation.

Nobiletin (ppm)	Growth rate (%)
0	100
25	72
50	60
100	52
200	45

ethanolic solution. The color formed in this reaction was orange and could be obtained on paper spots as well; the ultraviolet-absorption spectrum showed maxima at 270 and 320 mμ. These characteristics are identical with those of crystalline tangeritin, 5,6,7,8,4′-pentamethoxyflavone (Fig. 1B; *7–9*).

The various fractions thus obtained from the methanol extracts of Dancy and other tangerine peels were tested in vitro for fungistatic activity toward *D. tracheiphila*. One milliliter of test solution was added to 9 ml of potato dextrose–agar medium. The mixture was sterilized, inoculated with *D. tracheiphila*, and incubated at 21°C for several days. The growth of colonies was examined every second day by measuring their diameters.

These tests clearly showed nobiletin to be the main fungistatic substance present in the peel. In one series of experimental studies of the growth of *D. tracheiphila*, the growth of 25 replicates, with nobiletin at 200 parts per million, averaged 50.6 percent of that of the controls. Under the same conditions the average growth in the tests in which authentic tangeritin (*8*) was used was 87.0 percent of that of the controls. These differences are highly significant ($P < .01$). Table 1 shows

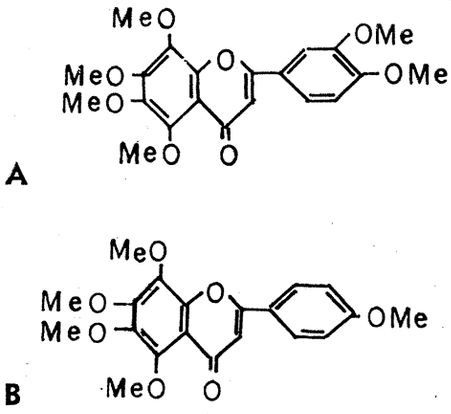

Fig. 1. Structural formulas of nobiletin (A) and tangeritin (B).

that a concentration of nobiletin as low as 25 ppm markedly inhibited growth of the fungus.

Examination of the structural formulas of the two compounds isolated (Fig. 1) suggests a possible connection between fungistatic activity and the presence of a methoxy group in position 3′ of the *B*-flavone ring, since the two structures are identical apart from this group.

Precipitates 1 and 3 stimulated fungal growth by 15 percent; when purified, both were identified as hesperidin (melting point, 258° to 262°C; absorption maxima at 286 and 330 mμ in 95-percent ethanol). This flavanone glycoside widely occurs in lemon and other varieties of citrus (*3*).

The results of my experiments in vitro led to a test with nobiletin in vivo. Sixteen 1-year-old, healthy, rough lemon seedlings, which are highly susceptible to mal secco, were inoculated with *D. tracheiphila* in the following manner. The fungus was grown on a liquid medium until the mycelium developed a dense mass of pycnidia containing fertile spores. This mass was disintegrated, and portions were introduced into cuts made in the main stems of the seedlings before the cuts were tightly covered with cellotape.

Starting immediately after the inoculation, 8 of the 16 seedlings were treated with a nobiletin extract (*10*) containing 100 ppm of nobiletin and 5 percent ethanol. This solution was administered continuously over a 3-month period as follows: a syringe needle was connected to a 100-ml plastic reservoir containing nobiletin solution. The point of the needle was introduced into the xylem of the main stem in an area about 10 cm below the inoculation site, resulting in continuous infusion of the nobiletin solution. Control seedlings were treated similarly but with the fungistat omitted. The results of one such experiment appear in Table 2.

After 4 weeks of treatment most of the control seedlings showed the first signs of mal secco. Within 6 weeks, six of the untreated controls died, and two died 1 week later. On the other hand, none of the nobiletin-treated seedlings died within 3 months, although two began to show signs of disease.

My data confirm the presence of both nobiletin and tangeritin in the inhibitive fraction described (*4*). These substances appear to exist in leaves,

Table 2. Effect of treatment with nobiletin on rough lemon seedlings inoculated with *D. tracheiphila*. Of 16 seedlings inoculated, half were treated with nobiletin (see text), while the remainder served as controls.

After inoculation (wk)	Seedlings affected (No.)	
	Controls	Treated
4	6*	0
6	2*	0
	6†	0
8	All dead	0
12		2*

* Chlorotic appearance of upper leaves. † Die-back of main stem, followed by death a few days later.

bark, fruit peel (*5*), and flowers of tangerines and clementines known to resist mal secco. A related compound, 5,6,7,3′4′-pentamethoxyflavone, recently isolated from orange peel (*11*), should be worth testing for fungistatic activity. Virtually nothing is known of the concentrations and possible seasonal variations of nobiletin in citrus plants; however, their absence from lemons and grapefruit susceptible to mal secco has been reported (*4*).

Pending confirmation of our data by large-scale field trials, we conclude that nobiletin is the natural compound that imparts resistance to the pathogenic fungus *D. tracheiphila* in some varieties of citrus. This finding sheds new light on resistance to mal secco and may point the way to new means of control.

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