Action of Oil in the Control of Citrus Greasy Spot

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ABSTRACT

Sprays of 1% oil-water emulsion applied to sweet orange (*Citrus sinensis*) leaves did not reduce germination and germ tube growth by ascospores of *Mycosphaerella citri* deposited 4 hr later on the treated lower leaf surface. However, oil sprays applied even as early as 56 days before inoculation reduced appressoria formation and the number of subsequent hyphal penetrations into the mesophyll. Hyphal penetration was also reduced by oil sprays applied 14 days after inoculation. The level of disease control, however, was always much greater than could be attributed solely to reduced hyphal penetration. Furthermore, oil sprays still controlled greasy spot even when applied after the fungus had invaded the spongy mesophyll. This suggests that the main action of oil in controlling greasy spot is by its inducing greater host resistance, thereby preventing or delaying hypertrophy of the spongy mesophyll.

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Additional key words: perithecial development, benomyl, copper fungicide.

Circumstantial evidence suggested that the partial replacement of oil sprays in Florida citrus groves by other pesticides, beginning in the late 1940's, led to increased severity of greasy spot caused by *Mycosphaerella citri* Whiteside. Thompson et al. (12) and Fisher (5) later confirmed that oil sprays applied during the summer reduced greasy spot. Spray oils meeting FC 435-66 specifications (10), applied as a 1% oil-water emulsion in June or July, are currently recommended for the control of greasy spot and certain citrus pests (6).

Cohen (4) reported that, although oil and tribasic copper sulfate gave similar control of greasy spot when applied in late June or July, oil was better than copper when applied from August to October. In recent studies (15) on spray timing in relation to disease epidemiology, oil gave similar control of greasy spot on the current year's spring flush of growth at all times of application from May to August, even though peak inoculum release occurred in June or July. In greenhouse tests on artificially inoculated plants, oil reduced greasy spot when applied either before or after inoculation (15).

The studies reported herein were designed to determine whether oil sprays can inhibit fungal development at any stage during the infection process, and whether oil has a direct action on pathogenesis unrelated to any effect on fungal growth. To provide additional clarification of these possible actions, the effect of oil in controlling greasy spot was compared with that obtained with sprays of a supposedly protectant copper fungicide, tribasic copper sulfate, and with that obtained with benomyl, the latter being selected because it had shown apparent systemic-eradicant action in previous tests (15).

MATERIALS AND METHODS.—Oil, containing 97.5% petroleum oils, meeting FC 435-66 specifications (10), was applied as a 1% oil-water emulsion. Tribasic copper sulfate (53% Cu) (TBCS) was applied at 1.5 lb./100 gal, and benomyl was applied as 0.5 lb. Benlate 50 W/100 gal. All sprays were applied to drip off and, except where otherwise stated, they were applied to both the dorsal and ventral leaf surfaces. After treatment, care was taken so that the foliage was not wetted during watering.

Experiments were carried out in the greenhouse on container-grown plants of sweet orange [*Citrus sinensis* (L.) Osbeck 'Pineapple'] and rough lemon [*Citrus limon* (L.) Burm.] that had been previously pruned to leave only two shoots. Data were obtained only from leaves that had reached full expansion not more than 4 weeks before inoculation or treatment, whichever occurred first. Four-plant replicates were used in all experiments.

For studies on the effect of oil on germination and germ tube growth, ascospores were obtained from perithecia produced on naturally infected decomposing sweet orange leaves. These leaves were collected from the ground in a citrus grove, held for 2 days at 100% relative humidity, air-dried for 2 hr, then sprayed with water and placed in a spore trap-impactor device as described by Brook (1). The ejected ascospores were impinged either directly onto the surface of microscope slides or onto leaf discs (10 mm in diam) affixed to the slides.

The plants were inoculated, as previously described (14), with a mixture of fragmented hyphae and conidia obtained from cornmeal agar cultures. They were then placed for 7 days in a clear polyethylene chamber and kept moist by a spraying with water once/day. All time intervals reported in this paper, from inoculation to treatment, commenced at the time the plants were removed from the moist chamber and replaced on the greenhouse bench.

Disease ratings were made only on the 20 uppermost leaves (10/shoot) on each plant. Estimates of the percentage leaf area diseased, as a measure of pathogenesis, included the yellowish green areas surrounding the greasy spots, as well as the necrotic areas themselves.

For studies on perithecial-producing capacity, 10 leaves (five/shoot) were picked from each plant and

placed flat between two layers of 0.5 mm-mesh hardware cloth. These leaves in the hardware cloth carriers were thereafter held on the greenhouse bench, dried first for 3-4 days, then wetted daily for 4-6 weeks until most of the perithecia had matured. Perithecia were too numerous to count, and an estimate of perithecial production was therefore obtained by a count of the number of squares/10 randomly selected 0.5 mm squares/leaf that contained perithecia. Data on perithecial-producing capacity proved useful for indicating whether treatments had any systemic-eradicant effects.

For histological studies, the penultimate leaf at the apex of each shoot was picked and fixed in Formalin:acetic acid:alcohol. Hyphae of *M. citri* within the host tissue were differentiated by a staining with Heidenhain's iron hematoxylin. Determinations of appressoria formation and hyphal penetration through the stomata, for limited distances into the mesophyll, were made on tangential sections, comprising the epidermis and a few underlying cells, cut freehand from four locations on the lower surface of each leaf. Counts were made, within a single field (0.45 mm in diam) on each section, of the number of stomata containing appressoria, and of the number of such stomata from which hyphae grew into the underlying mesophyll tissue. For observation of the depth of hyphal penetration into the spongy mesophyll, four pieces of tissue, each ca. 5 mm square, were removed from each leaf, fixed and embedded in paraffin wax, then sectioned transversely at 10 μ thickness with a rotary microtome.

RESULTS.—*Effect of oil sprays on ascospore germination and germ tube growth.*—Ascospores were deposited on the surface of clean microscope slides and onto slides smeared thinly with spray oil concentrate. The spore-laden slides were sprayed lightly with water from an atomizer, then held at 100% relative humidity for 24 hr. Spore germination and mean germ tube lengths after 24 hr were 80.2% and 34.6 μ, respectively, on clean slides, and 79.3% and 18.0 μ on oil-smeared slides.

For a determination of the effect of oil on ascospore germination and germ tube growth on the surface of oil-sprayed sweet orange leaves, leaf discs were removed 4 hr after spraying and attached to microscope slides with petrolatum, leaving the lower leaf surface exposed. Ascospores were immediately impacted on the leaf discs, which were then covered with a film of water from an atomizer and held at 100% relative humidity for 24 hr. Germination and mean germ tube lengths by ascospores deposited on leaf discs from nontreated leaves were 88.7% and 37.8 μ, as compared with 90.5% and 37.4 μ for ascospores germinated on discs obtained from oil-sprayed leaves.

Effect of oil and fungicide sprays applied before inoculation on fungal development and pathogenesis.—Sweet orange plants were sprayed with oil, benomyl, or TBCS at 56 or 28 days before inoculation with *M. citri*. Leaf samples for histological examination were picked at 63 days, and for perithecial development at 191 days, after inoculation.

The results (Table 1) showed that preinoculation sprays of benomyl and TBCS were both highly effective in protecting leaves from infection by *M. citri*. Oil also reduced the number of hyphal penetrations into the leaf, but this effect alone was insufficient to account for the low disease-severity ratings. On oil-sprayed leaves, perithecial ratings were much higher than on those sprayed with benomyl or TBCS, but lower than on the inoculated but nonsprayed controls.

Effect of oil and fungicide sprays applied after inoculation and infection on fungal development and pathogenesis.—The results of one experiment in which sweet orange leaves were sprayed with oil, TBCS, or benomyl at different times after inoculation are shown in Table 2. Leaves for histological study and for perithecial development were picked at 62 and 192 days, respectively, after inoculation.

TABLE 1. Appressoria formation, hyphal penetration into mesophyll tissue, disease severity and perithecial-producing capacity on sweet orange leaves sprayed before inoculation with *Mycosphaerella citri*

Treatment	Days between spraying and inoculation	Stomata with appressoria /10 mm^2	Hyphal penetrations into mesophyll /10 mm^2	% Appressoria from which hyphae grew into mesophyll	% Disease severity 121 days after inoculation[a]	% Area of incubated leaves with perithecia[b]
Control[c]		234 z[d]	108 z	44.9 x	26.1 y	83.0 z
Oil	28	98 y	31 y	34.1 x	2.5 x	50.5 y
	56	95 y	21 y	19.2 x	2.8 x	39.5 y
Tribasic copper sulfate	28	4 x	2 x	NC[e]	0.2 x	5.0 x
	56	6 x	2 x	NC	0.2 x	ND[f]
Benomyl	28	0 x	0 x	NC	0 x	0.2 x
	56	0 x	0 x	NC	0 x	ND

[a] Disease severity was based on the percentage leaf area showing necrotic and chlorotic symptoms.
[b] Based on number of squares per 10 randomly selected 0.5-mm squares/leaf that contained perithecia.
[c] Control not treated but inoculated.
[d] Numbers followed by the same letter are not significantly different at $P = .05$.
[e] NC = not calculated because appressoria too few to provide meaningful data.
[f] ND = no data obtained.

TABLE 2. Hyphal penetration into the mesophyll, disease severity and perithecial-producing capacity on sweet orange leaves sprayed after inoculation with *Mycosphaerella citri*

Treatment	Days between inoculation and spraying	Stomata with appressoria /10 mm^2	Stomata from which hyphae grew into mesophyll /10 mm^2	% Appressoria from which hyphae grew into mesophyll	% Disease severity: days after inoculation[a]		% Area of incubated leaves with perithecia[b]
					130	186	
Control[c]		183 y[d]	94 y	50.7 y	17.9 x	25.3 w	95.2 w
Oil	14	177 y	35 z	19.9 z	0.6 z	7.8 y	70.5 x
	28	198 y	104 y	48.9 y	2.5 z	10.5 xy	86.2 w
	41	ND[e]	ND	ND	3.7 yz	13.0 xy	83.5 w
Tribasic copper sulfate	14	114 z[f]	41 z	NC[g]	11.8 xy	15.7 x	85.2 w
	28	ND	ND	ND	15.7 x	24.0 w	ND
Benomyl	14	94 z[f]	2 z	NC	0.0 z	0.2 z	1.2 z
	28	216 y	116 y	52.9 y	4.1 yz	6.6 yz	15.5 y
	41	ND	ND	ND	7.7 yz	9.7 xy	21.5 y

[a] Disease severity based on percentage leaf area showing necrotic and chlorotic symptoms.
[b] Based on number of squares per 10 randomly selected 0.5-mm squares/leaf that contained perithecia.
[c] Inoculated but not treated.
[d] Numbers followed by the same letter are not significantly different at $P = .05$.
[e] ND = no data obtained.
[f] Appressoria were dislodged during preparation of sections and counts were therefore artificially low.
[g] NC = not calculated.

The appressoria of *M. citri* are produced only in the outer stomatal chambers (16). They are persistent and continue to enlarge for several weeks after their initiation, and thereby become increasingly securely anchored within the outer stomatal chamber. The low numbers of appressoria recorded in Table 2 for the 14-day treatments with benomyl and TBCS were due to inhibition of further appressorial growth, at a time when some of these bodies were still too small to remain seated in the outer stomatal chamber during the preparation of the material for histological examination. Only those appressoria that remained within the stomatal opening could be reliably included in the count.

TBCS reduced disease severity when applied 14 days after inoculation, probably due to death of the appressoria by direct contact with the fungicide, before hyphae had grown far enough into the leaf tissue to escape the reach of this nonsystemic fungicide. TBCS applied 28 days after inoculation did not reduce either the amount of hyphal growth into the mesophyll or disease severity.

Apparently, benomyl applied 14 days after inoculation almost completely prevented further fungal development, to judge from the fact that very few perithecia formed on the leaves after incubation. This effect also occurred, although to a lesser degree, when benomyl was applied at 28 or 41 days after inoculation, after the hyphae had reached the mesophyll.

Although oil and TBCS applied 14 days after inoculation reduced hyphal penetration into the mesophyll to similar extents, less greasy spot developed on leaves sprayed with oil than with TBCS. Oil applied 28 days after inoculation apparently had no effect on fungal growth within the leaf, yet these sprays still inhibited or retarded greasy spot development.

Perithecial production provided a useful indication of fungal survival in treated leaves. But, considering that perithecia are formed near the leaf surface (16), it was questionable whether such data provided a reliable estimate of the extent of fungal development within the deeper lying parts of the spongy mesophyll. The most consequential reaction to invasion by *M. citri* occurs in the spongy mesophyll (16), and only limited observation on hyphal ramification within this tissue was possible in the tangential sections. It became necessary, therefore, to prepare transverse sections through the leaf to examine more thoroughly the extent of the fungal invasion. Because of the relatively few hyphal penetrations into the sweet orange leaves, a proportionately large and unmanageable number of sections would have been required to provide any useful quantitative data on this subject. Therefore, another postinoculation spraying experiment was carried out; this time on rough lemon leaves, because these had been found to be more susceptible than sweet orange leaves to fungal invasion. They were also more convenient for studies on pathogenesis because of a shorter incubation period than on sweet orange leaves. Results of the studies on rough lemon plants are shown in Table 3. Leaf samples for histological examination were taken at 53 days; and for perithecial-producing capacity, at 83 days after inoculation. The effects of the 7-day postinoculation sprays of oil, TBCS, and benomyl on the pathogenesis of rough lemon leaves were essentially similar to those reported in Table 2 for the 14-day postinoculation sprays on sweet orange leaves. The data from the experiment with rough

lemon showed that the hyphal density throughout the spongy mesophyll was in proportion to the number of hyphae that in the tangential sections were seen to have reached the mesophyll tissue immediately above the lower epidermis. Thus, the 7-day postinoculation oil treatment apparently did not inhibit hyphal growth in the deeper lying portion of the mesophyll, but only reduced the number of initial penetrations.

Effect of oil applied to prescribed parts of leaf on greasy spot control.—Seven days after inoculation of rough lemon leaves with *M. citri*, a 1% oil-water emulsion was applied to defined parts of the leaf either with a brush or by a spray. When the latter method of application was used, the nonsprayed areas were protected before spraying by an aluminum foil cover.

Oil only controlled greasy spot when it was applied to the lower leaf surface. In one test in which disease ratings were made 53 days after inoculation, disease severity was 66.0% when only the upper surface was sprayed and 11.9% when only the lower surface was sprayed, compared with 68.3% on inoculated but nontreated checks. The dividing line between healthy and diseased tissue followed closely the margin of the treated areas, and the effect of the oil in reducing greasy spot did not extend more than 5 mm beyond the point of application.

DISCUSSION.—The results indicated that germination and germ tube growth by ascospores reaching the surface of oil-sprayed leaves, even as soon as 4 hr after spraying, would not be appreciably affected by the presence of oil residues. Feasibly, however, part of the action of oil in controlling greasy spot under field conditions could be through some interference, as observed in the greenhouse experiments, with appressoria formation and hyphal penetration into the leaf. A similar suppression of appressoria formation has been reported for *Mycosphaerella musicola* Leach on oil-sprayed banana leaves (11).

The results of the greenhouse experiments showed, however, that although some reduction in greasy spot severity by oil sprays, particularly when applied before inoculation, could be attributed to reduced hyphal penetration, the major effect was probably due to some other action. Substantial reduction in disease severity still occurred when oil was applied at 41 days after inoculation, long after the fungus had reached the spongy mesophyll. There were some indications that oil acted partly by only delaying pathogenesis, because from 130 to 186 days after inoculation, disease severity increased more rapidly on oil-sprayed leaves than on leaves sprayed with benomyl. The latter material apparently had some eradicant action which continued, albeit to a decreasing degree, even when it was applied after hyphae had reached the spongy mesophyll.

There is usually a long delay between the time when hyphae reach the large air spaces of the spongy mesophyll and the time when hypertrophy commences, and on some leaves the infection can remain latent indefinitely (16). This suggests that citrus leaves, under certain environmental conditions, have a natural resistance to the effects of hyphae present in the intercellular spaces. Oil might act by promoting a similar resistance in leaves that were previously naturally susceptible to greasy spot.

The mode of action by which oil controls greasy spot of citrus could be similar to that suggested for the action of oil in controlling leaf spot of bananas caused by *Mycosphaerella musicola*. In a review of this subject, Calpouzos (2) considered it likely that oil exerts a therapeutic action not directly on the pathogen, but rather through an alteration in the physiology of the host. He drew attention to the similarities between greasy spot of citrus and leaf spot of bananas in that both are controllable by oil, both have long incubation periods, and both may be affected by light intensity. Although high light intensity has been shown to favor the development of

TABLE 3. Hyphal penetration into spongy mesophyll, disease severity and perithecial-producing capacity on rough lemon leaves sprayed 7 days after inoculation with *Mycosphaerella citri*

Treatment	Stomata with appressoria /10 mm^2	Stomata from which hyphae grew into mesophyll /10 mm^2	% Appressoria from which hyphae grew into mesophyll	% Incidence of hyphae in spongy mesophyll[a]	% Disease severity 53 days after inoculation[b]	% Area of incubated leaves with perithecia[c]
Control[d]	1,170 x[e]	894 x	76.4 y	82.3 x	68.3 w	67.0 y
Oil	989 y	446 y	44.5 z	54.8 y	11.9 y	55.2 y
Tribasic copper sulfate	844 y	398 y	NC[f]	51.1 y	52.2 x	62.6 y
Benomyl	461 z	11 z	NC	7.3 z	0.5 z	6.4 z

[a] Based on the presence of hyphae in 16 blocks of leaf tissue, each 50-μ thick × 450-μ wide, represented by five consecutive tissue sections, each 10-μ thick, and a microscope field of 450-μ in diam.

[b] Disease severity based on percentage leaf area showing necrotic and chlorotic symptoms.

[c] Based on number of squares per 10 randomly selected 0.5-mm squares/leaf that contained perithecia.

[d] Inoculated but not treated.

[e] Numbers followed by the same letter are not significantly different at $P = .05$.

[f] NC = not calculated because many appressoria were dislodged during preparation of sections for histological examination.

banana leaf spot (3), experimental support for any similar effect of light on the development of greasy spot is currently lacking. Observations in Florida citrus groves that greasy spot is usually more severe on leaves exposed to direct sunlight suggest, however, that light may also play a role in promoting the development of this disease.

Calpouzos (2) drew attention to the fact that banana leaves in the shade have a lower rate of photosynthesis than those in full sunlight; and that photosynthesis, as reported by Riedhart (8), can also be retarded by oil. He, therefore, suggested that oil sprays may induce a disease resistance in exposed banana leaves by modifying the physiology of the leaf tissue to resemble that of shaded leaves.

Oil sprays also reduce photosynthetic rates on citrus leaves, but only when applied to the lower leaf surface (7). Because stomata are almost entirely restricted to the lower leaf surface of citrus leaves, McMillan & Riedhart (7) suggested that oil has to enter the leaf through the stomata to reduce photosynthetic activity. Riehl & Wedding (9) showed that the principal effect on photosynthesis occurs in oil-soaked areas of citrus leaves, and they suggested that inhibition of photosynthesis was the result of interference with gaseous exchange caused by the presence of spray oil within the leaf. The fact that greasy spot was only controlled when oil sprays were applied to the lower leaf surface suggests that oil also has to penetrate the leaf before there can be any control of this disease.

The effects of oil in reducing photosynthetic rates, in suppressing or delaying greasy spot development, and in interfering with stomatal penetration by hyphae of *M. citri* may be associated with the ability of this hydrophobic substance to penetrate citrus leaves, thereby forming a barrier in the stomata and in the intercellular spaces against normal moisture movement and gaseous exchange. The action of oil in controlling greasy spot might, therefore, be due to its special physical rather than chemical properties. This suggestion is also supported by the work of Trammel & Simanton (13), who reported that improved greasy spot control by different oils is related more to increasing distillation temperature than to changes in hydrocarbon composition. Distillation temperature is a measure of oil volatility: the higher the value, the longer the oil persists. Effective greasy spot control probably, therefore, depends on the oil remaining within the intercellular spaces for a long enough time.

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