Effect of Urea, CaCO₃, and Dolomite on Pseudothecial Development and Ascospore Production of *Mycosphaerella citri*

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ABSTRACT

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Citrus greasy spot, caused by *Mycosphaerella citri*, produces lesions on leaves, followed by premature defoliation, and rind blotch on fruit. Ascospores produced in leaf litter represent the major source of inoculum. The effect of treatment of leaf litter with urea, CaCO₃, or dolomite on the development of pseudothecia and ascospore production was evaluated. In laboratory experiments, one urea application reduced production of pseudothecia and ascospores by up to 90%, but did not affect time of production of pseudothecia or ascospores or rate of leaf decomposition. Two applications of urea delayed leaf decomposition. As the rates of CaCO₃ or dolomite were increased, pseudothecial incidence, density, time to ascospore production, and total numbers of ascospores decreased and the rate of leaf compostion increased. Immature pseudothecia on leaves treated with urea or CaCO₃ degenerated and produced fewer ascospores per pseudothecium. The results observed in microplot studies in the field were similar to those observed in laboratory experiments. The number of days to pseudothecia and ascospore production and the pseudothecial incidence and density were negatively related to the rate of CaCO₃ or dolomite applied. Application of CaCO₃, dolomite, or urea to leaf litter can reduce inoculum and be useful in an integrated program of greasy spot management.

Greasy spot, caused by *Mycosphaerella citri* Whiteside, produces lesions on mature citrus leaves which result in premature defoliation of the tree (14). Excessive leaf loss debilitates trees and reduces vigor and fruit yield (14,21). Greasy spot affects all citrus but is most severe on grapefruit and some grapefruit hybrids. In addition to foliar symptoms, *M. citri* can infect stomates on fruit, producing necrotic lesions which reduce the acceptability of fruit for the fresh market. Greasy spot is a significant problem on citrus in Florida and the Caribbean Basin and may require from one to three applications of fungicide for adequate control (14,15) on nearly all groves.

Pseudothecia and ascospores of *M. citri* are produced in decomposing leaf litter on the grove floor (14,17,19,20). Ascospores are airborne and are deposited on the abaxial side of the leaf, where they germinate, and the fungus grows epiphytically.

After several weeks, *M. citri* penetrates the stomates and invades the mesophyll. Symptoms often do not develop until 3 to 4 months after infection. In Florida, most of the infection occurs during the summer rainy season, the symptoms appear from November to January, and most of the defoliation follows in February to March (14,17). Peak ascospore release may occur from March to July, depending on the location (16,19). However, considerable infection may occur in late summer when ascospore numbers are low but when environmental conditions are favorable.

Frequent wetting of the leaf litter accelerates and maximizes production of pseudothecia and ascospores (11) and has resulted in earlier peaks in ascospore production (16). Changes in the Florida citrus industry have tended to exacerbate greasy spot problems. After the freezes of the 1980s, the industry moved southward, where temperatures are warmer and more favorable for greasy spot. Currently, herbicides, rather than tillage, are used for weed control. Thus, in the past, cultivation buried a portion of the inoculum and weed growth interfered with ascospore dispersal. Groves now are irrigated largely by microsprinklers, rather than by overhead irrigation. When rainfall is low, groves are irrigated three times weekly with microsprinklers; whereas, previously, they were watered only every 2 weeks. As a result, leaves fall on a uniform flat surface under the tree that is not cultivated, and leaves are moistened frequently, which favors

maximal production of pseudothecia and there are no weeds to interfere with ascospore dispersal to the tree canopy.

Reduction of ascospore inoculum by treatment of leaf litter has been used successfully in the control of apple scab caused by *Venturia inaequalis* (Cooke) G. Winter. Treatment of the leaf litter with urea and other nitrogen fertilizers, application of lime, or shredding of the litter have substantially reduced inoculum production and disease severity the following season (2,9,10,13). Treatment of leaf litter with chemicals such as phenylmercuric chloride (3) and application of fungal antagonists (4) also have been successful in reducing ascospore inoculum.

Leaf litter treatments for control of citrus greasy spot have been investigated only to a limited extent. Whiteside (18) found that benomyl treatment of decomposing leaves reduced ascospore production. Application of copper fungicides, captafol, or chlorothalonil were ineffective in reducing inoculum production.

The purpose of this study was to assess the effects of urea, CaCO₃, and dolomite on pseudothecial development and ascospore production to evaluate the potential of these products to control greasy spot in the field.

MATERIALS AND METHODS

Laboratory experiments. Mature leaves of grapefruit (*Citrus paradisi* Macfad.) severely affected by greasy spot were collected directly from trees in groves that had not been treated with fungicides. Leaves were air dried in the laboratory at ambient temperature (approximately 23 to 27°C). Most laboratory experiments were conducted in plastic containers, 42 cm long by 29 cm wide by 15 cm deep, to which field soil had been added to a depth of 5 cm. Soil moisture was maintained at near-field capacity, about 35% moisture (wt/vol), throughout the course of the experiment by adding water to maintain a constant weight. In all, 100 leaves were added to the soil surface in each plastic container. Treatments were applied to three replicate containers. Containers were maintained on the laboratory bench under ambient conditions for the duration of the experiments.

In experiment 1A, dried leaves were treated with CaCO₃, urea, or both and compared with a water-treated control. Calcium carbonate was applied by sprin-

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klung the powder over the leaves in plastic containers at 250 g/m². Urea was applied as a 5% solution (wt/vol) and sprayed over the surface of the leaves at 40 g/m². One treatment included both CaCO₃ and urea. In experiment 1B, CaCO₃ and urea were applied to leaves in the same manner as in experiment 1A. A single urea spray, two sprays 15 days apart, and a urea + CaCO₃ treatment were compared with a water-treated control. In experiment 1C, CaCO₃ or dolomite (49% CaCO₃, 36% MgCO₃; Soil Doctor, Polk City, FL) was applied dry at 125, 250, or 500 g/m² each and compared with a water-treated control. In experiments 1A, 1B, and 1C, leaves were placed on the soil surface for 2 weeks prior to application of the treatments. Leaves were sprayed with water three to four times per week to provide favorable conditions for pseudothecial development. Once pseudothecia were mature, all leaves were soaked for 30 min, once per week, to assay ascospore release, but were not otherwise wetted.

In laboratory experiments 1A, 1B, and 1C, the percentage of leaves with pseudothecial initials and the density of pseudothecia on leaves were assessed biweekly under a dissecting microscope with epillumination at $\times 10$. Time required for pseudothecial production was recorded in all treatments. The density was rated using a 0-to-10 rating scale where 0 = none, 1 = 1 to 5%, 2 = 6 to 10%, 3 = 11 to 15%, 4 = 16 to 20%, 5 = 21 to 25%, 6 = 26 to 30%, 7 = 31 to 35%, 8 = 36 to 40%, 9 = 41 to 45%, and 10 = >50% leaf area covered with pseudothecia. Pseudothecia were counted by arbitrarily cutting two 7-mm-diameter leaf discs from each of five arbitrarily selected leaves in each replicate. Leaf disks were cleared by soaking in a mixture of two parts 95% ethanol to one part lactophenol prior to mounting on microscope slides for examination. Asco-

spores were produced and collected by soaking all leaves in water for 1 h and then placing them in a wind tunnel apparatus designed by Whiteside (18) and modified by Mondal and Timmer (11). Ascospores were collected and counted as described previously (11). Ascospores were collected weekly from each sample and expressed as a cumulative total of all collections. The stage of leaf decomposition was assessed in most experiments. Leaves were rated on a scale of 0 = not decomposed, still firm; 1 = partially decomposed, flexible, still intact; 2 = moderately decomposed, some loss of lamina; and 3 = highly decomposed, skeletonized leaves.

In experiments 2A and B, the effect of application of CaCO₃ or urea on the development and maturation of ascospores on leaves with pseudothecial initials was determined. Dried leaves with greasy spot comparable to those used in experiments 1A to C were placed in plastic mesh bags and placed on the floor of a grapefruit grove for 25 to 30 days under field conditions until pseudothecia had formed. Air-dried leaves with pseudothecia were retrieved from the bags and soaked for 30 min in 5% CaCO₃, 5% urea, a combination of both materials, or water. Treated leaves were placed on moist paper towels under ambient conditions in the laboratory. Leaves were examined weekly and assessment of treatments made when most of the pseudothecia in the control were mature. Treatments were replicated on three sets of leaves in experiment 2A. Five leaves were selected arbitrarily from each replicate of each treatment and 50 pseudothecia per leaf were examined. In experiment 2B, treatments were replicated on five sets of leaves, five leaves were selected arbitrarily from each replication, and 10 pseudothecia per replicate were examined.

Individual pseudothecia were picked from the leaves and squash mounted in lactophenol-acid cotton blue following the procedures of Gadoury and MacHardy (6) to assess the stage of pseudothecial maturity. Pseudothecia were examined at $\times 200$ or $\times 400$ and scored as immature (no ascospores or lumen of pseudothecia filled with only pseudoparaphyses or with pseudoparaphyses and immature asci, mostly not stained), mature (stained and septate ascospores inside asci), or degenerated (lumen of pseudothecia filled with granular globose structures and either asci or ascospore-like structures) (6,11).

Treatments in experiment 1A and B and 2A and B were compared using analysis of variance and the means were separated by the Waller-Duncan *k*-ratio *t* test, $P \leq 0.05$. Linear regression analyses were used to determine the response of pseudothecia and ascospore production measurements to the rate of CaCO₃ and dolomite.

Field experiments. To confirm the results obtained in laboratory studies, similar experiments were conducted in microplots located on the floor of a grapefruit grove in Lake Alfred, FL. Wooden frames, 50 by 50 cm in size, were placed on the soil and 200 leaves, collected and prepared by the same methods as in laboratory experiments, were placed on the soil surface in each plot. Frames were covered with plastic mesh with 2-by-2-cm openings to contain the leaves within the frame. Leaves were left in the field for 2 weeks prior to treatment. In one experiment, urea and CaCO₃ were applied in the same manner as in the laboratory experiments. Treatments were (i) a single application of urea; (ii) two applications, the second 15 days after the first; and (iii) an application of urea followed immediately by an application of CaCO₃ at 250 g/m². In the second experiment, CaCO₃ and dolomite lime were applied at 125, 250, or 500 g/m² each and

Table 1. Effect of CaCO₃ and urea on pseudothecia development and ascospore production of *Mycosphaerella citri* in grapefruit leaves under laboratory conditions^v

Treatments [*]	Rate/m ²	Pseudothecia ^w				Ascospores		Leaf decomposition ^z
		Days to formation	Incidence (%)	Density	No./cm ² of leaf	Days to first discharge	Total no./leaf ($\times 10^4$) ^y	
Experiment 1A								
CaCO ₃	250 g	12 b	67.6 ab	2.5 b	50.8 b	28 c	19.3 b	3 a
Urea	40 g	28 a	58	1.1 d	15.6 d	49 a	2.8 d	2.1 bc
CaCO ₃ + Urea	250 g + 40 g	16 b	69 ab	1.5 c	40.3 c	42 b	8.5 c	2.6 ab
Untreated	...	23 a	71.6 a	4.5 a	82 a	49 a	38.9 a	2 c
Experiment 1B								
Urea, one spray	40 g	...	10 c	0.8 b	...	62 b	1.2 b	1.7 c
Urea, two sprays	80 g	...	6 c	0.1 c	...	87 a	0.4 c	1.0 d
Urea + CaCO ₃	40 g + 250 g	...	25 b	0.6 b	...	53 c	1.7 b	2.1 a
Untreated control	...	...	80 a	1.7 a	...	63 b	16.5 a	1.9 b

^v Mean separation by Waller-Duncan *k*-ratio *t* test ($P \leq 0.05$). Values within a column followed by the same letter are not significantly different.

^w Incidence represents the percentage of the leaves with pseudothecia; pseudothecial density was estimated using a 0-to-10 rating scale, where 0 = no pseudothecia and 10 = >50% leaf area with pseudothecia.

^x Grapefruit leaves naturally infected with *M. citri* collected and air dried for 2 to 3 days at room temperature (≈ 23 to 27°C) were placed on soil in plastic containers (42 by 29 by 15 cm) prior to treatments.

^y Cumulative total of weekly collections.

^z Leaf decomposition was recorded using a 0-to-3 scale, where 0 = undecomposed leaf without pseudothecia and 3 = highly decomposed and skeletonized leaves with no further ascospore discharge.

compared with a nontreated control. Pseudothecial development and ascospore production were measured in the same manner as in the laboratory experiments. Ascospore production was the cumulative total of all ascospores produced in 10 samples collected at approximately 7- to 10-day intervals.

The significance of treatment effects was determined by analysis of variance and treatment means separated by the Waller-Duncan k -ratio t test, $P \leq 0.05$. Linear regression analyses were used to determine the response of pseudothecia and ascospore production measurements to the rate of CaCO_3 and dolomite.

RESULTS

Laboratory experiments. In experiment 1A, application of CaCO_3 or urea significantly affected the development of pseudothecia and production of ascospores (Table 1). Treatment with CaCO_3 significantly reduced the time required for production of pseudothecia and ascospores, whereas urea had no effect. Both CaCO_3 and urea reduced the density of pseudothecia, but only urea reduced pseudothecial incidence. All treatments reduced ascospore numbers and the total ascospore production on urea-treated leaves was less than 10% of that from the control. In spite

Fig. 1. Ascospore production of *Mycosphaerella citri* in grapefruit leaves treated with CaCO_3 or urea under laboratory conditions. A, Pattern of ascospore discharge, and B, cumulative production of ascospores. Bars are standard errors of the mean.

Fig. 2. Effect of rate of application of CaCO_3 and dolomite on pseudothecial development and ascospore production by *Mycosphaerella citri* and decomposition of grapefruit leaves under laboratory conditions. A, Pseudothecial incidence, the percentage of leaves with pseudothecia present, B, pseudothecial density on a scale of 0 to 10, C, days to first ascospore release, D, total ascospore discharge from collections at 7-day intervals, and E, degree of leaf decomposition on a scale of 0 = undecomposed to 3 = highly decomposed, skeletonized. Regressions were significant at $P \leq 0.05$ (*) or $P \leq 0.01$ (**).

of the fact that CaCO₃ accelerated pseudothecia and ascospore production alone or in combination with urea (Fig. 1), total numbers were significantly less than in the control (Table 1). Leaves treated with CaCO₃ decomposed more rapidly than control leaves, but decomposition of those treated with urea was unaffected.

In experiment 1B, all treatments, including urea, significantly reduced the incidence of leaves with pseudothecia, density of pseudothecia on decomposing leaves, and total ascospore production. Two sprays of urea delayed the time to first ascospore discharge compared with all other treatments. Leaf decomposition was delayed by application of urea and accelerated by application of CaCO₃. In experiment 1C, the rate of application of CaCO₃ or dolomite was linearly related to all of the pseudothecia development variables, ascospore production, and leaf decomposition (Fig. 2). Coefficients of determination were high and significant in all cases. Increase in application rates decreased pseudothecial incidence, density, days to ascospore production, total ascospore production, and increased the rate of leaf decomposition.

In experiment 2A and B, pseudothecial development on leaves treated with CaCO₃, urea, or both was compared with the water-treated control. Most of the treated pseudothecia never properly ma-

tured and did not produce ascospores (Table 2). Those pseudothecia classed as "degenerated" were aborted and were not pseudothecia that had collapsed following ascospore release, but still contained malformed asci and ascospore-like structures. In most cases, the leaves treated with urea or the combination of urea and CaCO₃ had higher numbers of degenerated pseudothecia and lower numbers of ascospores per pseudothecium in both experiments (Table 2).

Field experiments. Similar results were observed when urea, CaCO₃, and dolomite treatments were applied to leaves in microplots in the field. The time to form pseudothecia and to first ascospore discharge was delayed by two applications of urea (Table 3). Addition of CaCO₃ to urea-treated leaves advanced the time to first ascospore release compared with any other treatment. Application of urea alone or in combination with CaCO₃ decreased the incidence and density of pseudothecia and the number of ascospores produced (Table 3). Leaf decomposition was accelerated by application of CaCO₃ with the urea and delayed by two urea sprays compared with the control.

In a second field experiment, the time to form pseudothecia and to first ascospore release decreased linearly as the rate of CaCO₃ and dolomite increased (Fig. 3).

However, the density of pseudothecia and the total number of ascospores produced decreased with increasing rates of both products as determined by linear regression analysis (Fig. 3). Leaf decomposition was not strongly affected by the rate of CaCO₃ or dolomite applied (Fig. 3E). Apparently, even low rates of these products accelerated decomposition.

DISCUSSION

Application of urea, CaCO₃, or dolomite to leaf litter substantially reduced the formation of pseudothecia and production of ascospores of *M. citri*. These materials also reduced ascospore production if applied after the pseudothecia had already formed. Urea was more effective in reducing pseudothecia than CaCO₃ or dolomite in most experiments. These treatments were effective in both laboratory and field experiments.

Urea has been highly effective in reducing pseudothecia and ascospore production of *V. inaequalis* leaves affected by apple scab (3,5,9,10). The effect of urea in those studies has been attributed to acceleration of decomposition of leaves by microorganisms (3,5,10). Burchill (2) found that application of urea and other nitrogenous compounds softened leaf tissue and made them more palatable to earthworms. Crosse et al. (5) suggested that the increase in pH

Table 2. Maturity of pseudothecia and production of ascospores of *Mycosphaerella citri* in pseudothecia-bearing grapefruit leaves after soaking in urea or CaCO₃ under laboratory conditions^y

Treatment ^z	Experiment 2A				Experiment 2B			
	Maturity of pseudothecia (% of total)				Maturity of pseudothecia (% of total)			
	Immature	Mature	Degenerated	Spores/pseud	Immature	Mature	Degenerated	Spores/pseud
CaCO ₃	8.0	14.1 b	56.2 b	194.5 b	0.0	38.4 b	61.6 b	155.7 b
Urea	0.7	8.9 c	90.3 a	48.6 d	0.0	3.2 d	96.8 a	13.0 d
Urea + CaCO ₃	0.0	16.8 b	83.2 a	91.5 c	0.0	13.6 c	86.4 a	55.2 c
Control	2.7	89.8 a	7.3 c	489.4 a	0.0	92.4 a	7.6 c	374.8 a
	N.S.				N.S.			

^y In Experiment 2A and B, 750 and 250 pseudothecia per treatment, respectively, were squashed in lactophenol-acid cotton blue, and microscopically examined as described by Gadoury and MacHardy (6). The pseudothecia were grouped as immature (no ascospores and lumen of pseudothecia filled with only pseudoparaphyses or with pseudoparaphyses and immature asci, mostly not stained), mature (stained and septate ascospores inside asci), and degenerated (lumen of pseudothecia filled with granular globose structures and either asci- or ascospore-like structures). Spores/pseud = ascospores per pseudothecium. Mean separation by the Waller-Duncan *k*-ratio *t* test (*P* ≤ 0.05). Values within a column followed by the same letter are not significantly different; N.S. = not significant.

^z Leaves were soaked for 30 min separately in water (control), in 5% urea (w/v), in 5% CaCO₃ or in a mixture of the products.

Table 3. Effect of urea on the pseudothecial development and ascospore production of *Mycosphaerella citri* in grapefruit leaves under field conditions^y

Treatment ^x	Rate/m ²	Pseudothecia ^w				Ascospores		
		Days to formation	Incidence (%)	Density	No./cm ² of leaf	Days to first discharge	Total no./leaf (×10 ⁴) ^y	Leaf decomposition ^z
Urea, one spray	40 g	30 b	43 b	0.8 bc	39.4 c	50 c	5.8 c	1.5 b
Urea, two sprays	100 g	38 a	12 c	0.1 c	9.3 d	80 a	2 d	1.1 c
Urea + CaCO ₃	40 g + 250 g	27 b	47 b	1.4 b	74.6 b	40 d	19 b	2.5 a
Control	...	25 b	96 a	3.1 a	107.4 a	60 b	51.4 a	1.6 b

^y Mean separation by Waller-Duncan *k*-ratio *t* test (*P* ≤ 0.05). Values within a column followed by the same letter are not significantly different.

^w Incidence represents the percentage of the leaves with pseudothecia; pseudothecial density was estimated using a 0-to-10 rating scale, where 0 = leaf without pseudothecia and 10 = >50% leaf area with pseudothecia.

^x Grapefruit leaves naturally infected with *M. citri* collected and air dried on the microplots (50 by 50 cm) under orchard conditions 15 days before application of treatments.

^y Cumulative total of weekly collections.

^z Leaf composition was recorded after 110 days of exposure using a 0-to-3 scale, where 0 = undecomposed leaf and 3 = highly decomposed and skeletonized leaves.

Fig. 3. Effect of rate of application of CaCO₃ and dolomite on pseudothecial development and ascospore production of *Mycosphaerella citri* and grapefruit leaf decomposition under field conditions. A, Days required for pseudothecial initials, B, days required for first ascospore discharge, C, pseudothecia per square centimeter of leaf tissue, D, total ascospore discharge from collections at 7-day intervals, and E, degree of leaf decomposition as measured using a 0-to-3 scale, where 0 = undecomposed and 3 = highly decomposed, skeletonized. Regressions were significant at $P \leq 0.05$ (*), $P \leq 0.01$ (**), or not significant (no asterisks).

following degradation of urea may have stimulated increases in fluorescent pseudomonads and other urease-active organisms which may have inhibited pseudothecial production. Work of Ross and Burchill (12) indicated that urea also may be directly toxic to *V. inaequalis*.

The inhibition of pseudothecial production and ascospore formation of *M. citri* by urea cannot be attributed to increased leaf decomposition. A single application of urea to citrus leaves did not significantly affect decomposition, and two applications actually delayed decomposition. The fact that urea affected already-formed pseudothecia indicates that it or its breakdown products may be directly toxic to *M. citri*.

Lime application reduced production of pseudothecia and ascospores of apple leaves affected by scab (10,13). Accelerated decomposition of leaves has been suggested as the mechanism of action of lime (10,13). Increased pH and Ca concentration have been shown to increase decomposition of litter in forest soils (8) and in acid peat bogs (7). In citrus, application of CaCO₃ and dolomite encourages rapid decomposition of leaf litter and, thus, reduces formation of pseudothecia. However, application of CaCO₃ to leaves that had pseudothecia already present inhibited pseudothecial maturation and ascospore formation. Thus, the effects of these materials may be more complex than their direct effect on decomposition.

The potential exists for utilizing the application of lime and urea in an integrated program for greasy spot control. Lime is applied to Florida citrus groves every 3 to 5 years to control soil pH (1). Such applications are too infrequent to have a major impact on greasy spot inoculum availability. However, growers could gain some benefit by applying lime when leaf litter is at a peak. Urea has more potential than lime as a beneficial treatment to reduce the inoculum of greasy spot. Up to 200 kg of N is applied per hectare per year to meet the nitrogen needs of mature trees (1). Rates used in this study are somewhat less than the annual N requirement for citrus groves. Urea could be applied as a boom spray in combination with herbicides or could be applied through microsprinkler irrigation. Thus, such treatments would add very little to the cost of production. However, the effect of multiple applications at lower rates needs to be evaluated to be able to treat all fallen leaves in a timely manner and to meet the needs of the tree and avoid leaching.

Treatments to reduce inoculum must decrease the spore load substantially in order to be effective. Much of the infection by *M. citri* occurs at times of the year when ascospore densities are low (16). The favorability of the environment for epiphytic growth of the fungus appears to be more important in determining disease severity than ascospore numbers. Thus, treatments

of leaf litter to reduce inoculum are unlikely to replace foliar applications of fungicides for disease control. Nevertheless, urea and occasional lime applications can be made at little or no additional cost to growers. Thus, perhaps properly timed applications of these products can at least reduce disease pressure and mitigate the increases observed with the use of chemical weed control and microsprinkler irrigation. Disease control with current fungicide programs then would be more effective and a higher degree of control could be attained with products such as petroleum oils.

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