# Suppression of the Symptoms of American Leaf Spot of Coffee with Calcium Hydroxide

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**ABSTRACT**

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Detached and undetached leaves of coffee were scratched and sprayed at 276 kPa with suspensions of calcium hydroxide containing 2 ml $L^{-1}$ of a spreader-sticker. The leaves received 0.04, 0.08, or 0.1 mg of calcium hydroxide $cm^{-2}$. The scratched points were inoculated with the gemmae of *Mycena citricolor* and the leaves were scored for lesion development 1 and 2 wk after inoculation. Calcium hydroxide spray significantly decreased the number and diameter of lesions and delayed their development, with lesion development being completely inhibited by a spray of 0.1 mg $cm^{-2}$. Scanning electron microscopy revealed numerous calcium oxalate crystals on lesions that developed on calcium hydroxide-sprayed and inoculated leaves as well as in calcium hydroxide-supplemented distilled water cultures of *M. citricolor*. It is suggested that the mode of action of calcium hydroxide in disease control is by way of neutralization of oxalic acid secreted by the pathogen. The results suggest that calcium hydroxide could control the disease on a field scale.

The American leaf spot or *ojo de gallo* is one of the most serious diseases of coffee in the Latin American countries (1,7). The causal fungus, *Mycena citricolor* (Berk. & Curt.) Sacc., a basidiomycete, incites brownish spots on coffee leaves and causes extensive defoliation. Young twigs and berries are also affected, causing berry drop.

The disease is severe during rainy seasons and the pathogen spreads by gemmae (1). The teleomorph is a basidioma, but the role of basidiospores in disease epidemiology is not known (1). The pathogen apparently survives the dry season in the form of a quiescent mycelium in dead fallen leaves.

The control of this disease has been primarily through the use of eradicant fungicides (2,3). This paper presents the results of laboratory studies with detached and undetached leaves showing that calcium hydroxide can effectively check the disease, indicating a strong potential for its use on a field scale. A brief report on part of this work was published earlier (8).

## MATERIALS AND METHODS

**Culture of *M. citricolor*.** A Costa Rican isolate of *M. citricolor* was kindly supplied by E. Vargas. It readily produces oxalic acid in culture and in the host tissue (5,6). The cultures were grown in petri plates containing potato-dextrose agar (PDA) supplemented with 2 g $L^{-1}$ of yeast extract at 22 C under continuous fluorescent lighting (approximately 30 $\mu Em^{-2}$ $sec^{-1}$). To maintain the virulence of the pathogen, it was occasionally transferred to coffee leaves and recovered in culture through the new gemmae formed on leaves. Gemmae from 3-wk-old PDA cultures were used for leaf inoculations.

**Coffee plants.** Coffee seedlings (*Coffea arabica* L. 'Caturra') were planted in 14-cm plastic pots containing sterilized soil, peat, and sand (1:2:1, v/v/v) and grown in a greenhouse at 20 C. During winter, the plants received supplementary lighting from high pressure sodium lamps at 155 $\mu Em^{-2}$ $sec^{-1}$ for 18 hr each day.

**Preparation of leaves for spraying with calcium hydroxide.** Young, turgid, and fully expanded leaves detached from 5-mo-old plants were used for spraying and subsequent inoculation with gemmae of *M. citricolor*. The detached leaves were washed in sterile double distilled water and each leaf was placed in a petri plate containing moistened sterilized vermiculite. The interveinal areas of both halves of the upper leaf surface were scratched at 4–6 points on each half with a hypodermic needle (9). In addition, 5-mo-old plants, each carrying four fully expanded leaves, were prepared for undetached leaf inoculations. Leaves on these plants were also scratched similarly.

**Spraying with calcium hydroxide.** Suspensions of calcium hydroxide ($Ca[OH]_2$) prepared in double distilled water were applied to the leaves from a RIC Spray Chamber (Research Instrument Co., R.R. 2, Guelph, Ontario, Canada) equipped with the spray nozzle E-8001 to deliver spray particles of approximately 350 $\mu$m diameter at 276 kPa pressure.

The leaves in each experiment were divided into three groups, each with five replicates. The individual groups were sprayed with calcium hydroxide at concentrations of 40, 80, or 100 g $L^{-1}$, containing the spreader-sticker Citowett Plus (BASF Canada, Inc., Rexdale, Ontario, Canada) at a final concentration of 2 ml $L^{-1}$. The approximate amounts of calcium hydroxide deposited were 0.04, 0.08, or 0.1 mg $cm^{-2}$ of leaf area, respectively. Leaves on groups of intact plants, three plants for each treatment, were sprayed in a fashion similar to that described above. Unless otherwise stated, all experiments were repeated twice with the inclusion of no-calcium hydroxide controls, but with 2 ml $L^{-1}$ of the spreader-sticker.

**Leaf inoculations with gemmae.** Inoculations of detached and undetached leaves were made according to the procedure of Tewari et al (9). Each scratched point on one half of the leaf was inoculated with 3–4 gemmae of *M. citricolor* in a droplet of sterile distilled water. The scratched points on the other half served as controls. The detached leaves were incubated at 20 C at a 12-hr light cycle at 28 $\mu Em^{-2}$ $sec^{-1}$. Plants with inoculated leaves were incubated in a dew chamber at 18 C day/12 C night cycles of 12 hr each, respectively. The plants received fluorescent lighting at 3 $\mu Em^{-2}$ $sec^{-1}$ for 12 hr each day at almost saturated humidity.

**Disease rating.** The leaves were scored for brownish lesion formation 1 and 2 wk after inoculation. The disease incidence was expressed as percent of lesions formed, out of the total number of scratched inoculated points. In addition, two orthogonal diameters of each lesion were measured and the mean was taken as the lesion diameter.

**Scanning electron microscopy (SEM) and energy dispersive x-ray microanalysis.** Crystal morphology of calcium hydroxide and calcium oxalate formed in culture as well as on detached calcium hydroxide-sprayed inoculated leaves was studied by SEM. An aliquot (0.2 ml) of 20 g $L^{-1}$ of calcium hydroxide suspension was placed on a carbon disk to which 0.2 ml of 12 g $L^{-1}$ of potato-dextrose (PD) broth (containing 2 g $L^{-1}$ of yeast extract) was added. This mixture was designated calcium hydroxide-broth. Three to five gemmae of *M. citricolor* were transferred aseptically to the calcium hydroxide-

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broth drop, and the disks, in triplicate, were placed in a petri plate, kept humid, and incubated at 22 C under continuous fluorescent illumination at 30 $\mu Em^{-2} sec^{-1}$. After 5 days, the disks were air-dried, mounted on stubs, and coated with gold for observations in the SEM. Calcium hydroxide-broth controls without gemmae were included. In addition, gemmae were incubated in 0.4-ml aliquots of calcium hydroxide in sterile water (2 g $L^{-1}$) and SEM observations were made 2 wk after incubation. Preliminary experiments indicated that growth from gemmae was very slow in 10 g $L^{-1}$ of calcium hydroxide without the addition of PD broth. Consequently, a lower concentration of calcium hydroxide was used in this control experiment.

The morphology of crystals formed on inoculated leaves previously sprayed with various concentrations of calcium hydroxide was examined by SEM, and energy dispersive x-ray microanalysis was made to detect major cations. Discrete energy levels characterized by K$\alpha$ and K$\beta$ emission peaks for each cation were recorded. Comparable inoculated, no-calcium hydroxide controls were included. Segments of leaves containing gemmae were cut and then processed for SEM according to Rao and Tewari (5). Micrographs were taken in a Cambridge Stereoscan 250 SEM equipped with Kevex Micro-X 7000 analytical spectrometer.

## RESULTS

**Calcium hydroxide spraying and disease development.** On detached leaves that were not sprayed with calcium hydroxide, 96% of scratched inoculated points developed lesions. Lesion development was completely inhibited at 0.1 mg $cm^{-2}$ calcium hydroxide spray concentration and the percent of lesions decreased with an increase in spray concentration (Table 1). Even in the treatment with the highest percent of lesions developed (0.04 mg $cm^{-2}$), the percent of lesions formed on detached and undetached leaves (32 and 36%) after 2 wk was significantly lower than that of the respective no-calcium hydroxide controls (96 and 79%).

During the second week, many lesions in no-calcium hydroxide controls expanded rapidly and coalesced into blotches, making it impossible to measure the lesion diameter, but only slight increases in lesion diameter took place on leaves treated with 0.04 and 0.08 mg $cm^{-2}$ of calcium hydroxide.

**Scanning electron microscopy and energy dispersive x-ray microanalysis.** Figure 1 shows scanning electron micrographs of coffee leaves and broth cultures demonstrating calcium hydroxide and calcium oxalate crystals. One week after treatment with 0.04 mg $cm^{-2}$, and in the presence of gemmae, calcium hydroxide was seen as amorphous

**Table 1.** Effect of calcium hydroxide on lesions incited by *Mycena citricolor* on detached and undetached leaves of coffee plants

| Concentration of calcium hydroxide (mg $cm^{-2}$) | Lesions | | | |
|---|---|---|---|---|
| | Detached leaves | | Undetached leaves | |
| | 1 wk | 2 wk | 1 wk | 2 wk |
| 0 | 8 ± 0.02[a] (96)[b] | ...[c] (96) | 11 ± 0.04 (79) | ...[c] (79) |
| 0.04 | 6 ± 0.05 (19) | 8 ± 0.07 (32) | 9 ± 0.06 (29) | 12 ± 0.07 (36) |
| 0.08 | 0 (0) | 3 ± 0.08 (3) | 7 ± 0.05 (12) | 11 ± 0.07 (14) |
| 0.1 | 0 (0) | 0 (0) | 0 (0) | 0 (0) |

[a] Mean lesion diameter in millimeters ± standard error.
[b] Numbers in parentheses denote the mean of percent number of lesions formed in three experiments for a total of 15 detached leaves, or in two experiments for a total of 24 undetached leaves on six plants.
[c] Lesions could not be measured because they had coalesced.

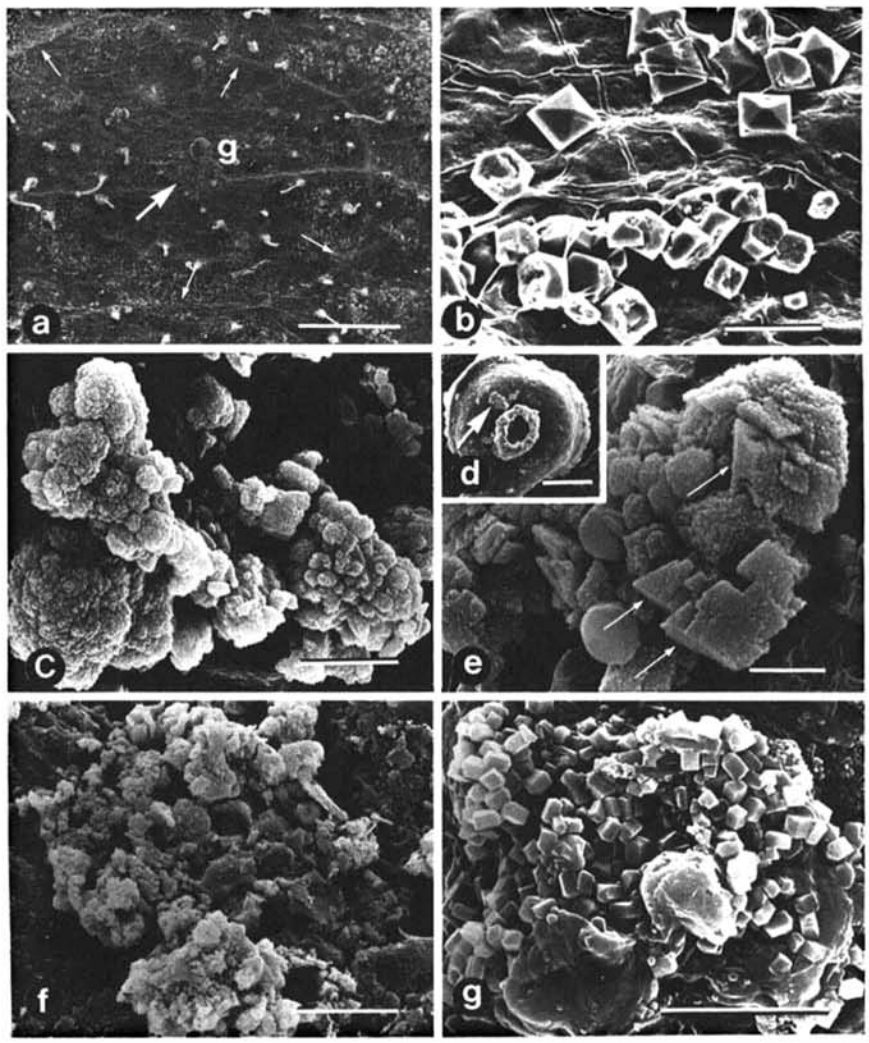


**Fig. 1. (A)** A low magnification scanning electron micrograph of a lesion on coffee leaf incited by *Mycena citricolor* after spraying with calcium hydroxide at 0.04 mg $cm^{-2}$. Note gemmae (g) in the center of the lesion. The large arrow points to an area shown at higher magnification in Figure 1B. The small arrows indicate the margin of the lesion (bar = 1,000 $\mu$m). **(B)** Area from the center of the lesion in Figure 1A. Note the hyphae of *M. citricolor* and bipyramids of calcium oxalate (bar = 40 $\mu$m). **(C)** Scratched control leaf with calcium hydroxide sprayed at 0.08 mg $cm^{-2}$. Note the amorphous aggregates of calcium hydroxide (bar = 6 $\mu$m). **(D)** An inoculated gemma on the leaf after spraying with calcium hydroxide at 0.08 mg $cm^{-2}$, showing crystals indicated by an arrow (bar = 100 $\mu$m). **(E)** Close-up of crystals on the surface of gemma in Figure 1D. Note delineated crystal faces (arrows) indicating transitional stages in calcium oxalate crystal formation (bar = 40 $\mu$m). **(F)** Amorphous calcium hydroxide aggregates in a potato-dextrose broth control (bar = 20 $\mu$m). **(G)** Numerous prismatic crystals of calcium oxalate in calcium hydroxide-broth cultures inoculated with gemmae (bar = 10 $\mu$m).

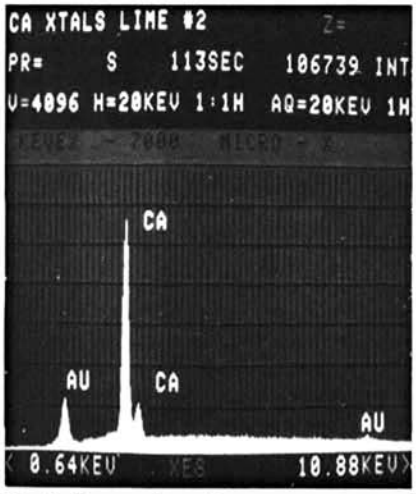


**Fig. 2.** Energy dispersive x-ray spectrum of calcium oxalate crystals formed on coffee leaf surface sprayed with 0.04 mg $cm^{-2}$ of calcium hydroxide and inoculated with *Mycena citricolor*. Note the characteristic K$\alpha$ and K$\beta$ emission peaks for calcium. The two gold peaks have resulted from coating of the sample for SEM.

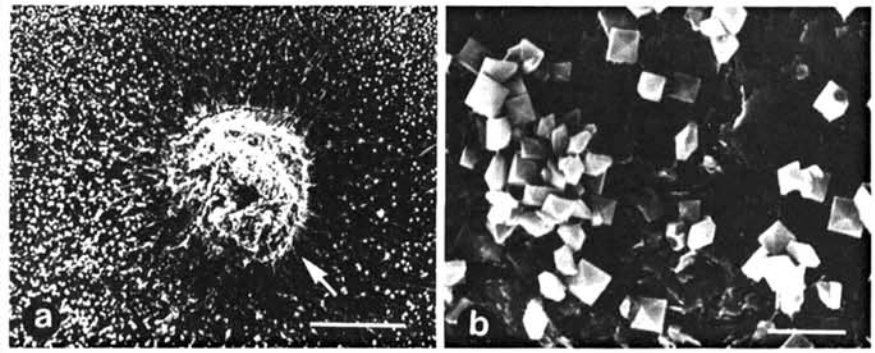


**Fig. 3. (A)** A low magnification scanning electron micrograph of a gemma of *Mycena citricolor* after incubation in 2 g $L^{-1}$ of calcium hydroxide in sterile water. Arrow indicates the head of a gemma with mycelium radiating from it. Note numerous crystals of calcium oxalate in association with the mycelium (bar = 200 $\mu$m). **(B)** A close-up view of crystals from (A). Note the bipyramids of calcium oxalate (bar = 20 $\mu$m).

aggregates distributed along the lesion margin (Fig. 1A), whereas the central part of the lesion where gemmae were placed (Fig. 1B) contained many bipyramids. These bipyramids were variable in size and exhibited crystal morphology characteristic of calcium oxalate polyhydrate-form (4–6). Corresponding controls that were not sprayed with calcium hydroxide, but inoculated with gemmae, also showed the polyhydrate form of calcium oxalate (5,6). However, calcium oxalate crystals on calcium hydroxide-sprayed inoculated leaves were strikingly more abundant than on the unsprayed controls. Energy-dispersive x-ray microanalysis indicated calcium as the only cation present in these crystals (Fig. 2).

Figure 1C shows a close-up view of scratched control leaves sprayed with calcium hydroxide at 0.08 mg $cm^{-2}$. Calcium hydroxide appeared as amorphous aggregates. However, in the case of 0.08 mg $cm^{-2}$ calcium hydroxide-sprayed and inoculated leaves, crystals with delineated faces resembling calcium oxalate were observed, usually on the surfaces of the inoculated gemmae (Fig. 1D,E). In the case of 0.1 mg $cm^{-2}$ calcium hydroxide-sprayed inoculated leaves, typical amorphous aggregates of calcium hydroxide only were observed. The calcium hydroxide-broth in which gemmae were incubated, however, showed prismatic calcium oxalate crystals (Fig. 1G) that were absent in the respective controls (Fig. 1F). Further, in experiments where gemmae were incubated in 2 g $L^{-1}$ of calcium hydroxide in sterile water, numerous bipyramids of calcium oxalate were observed in association with the mycelium from germinating gemmae (Fig. 3A,B).

Scanning electron microscopic studies revealed the formation of calcium oxalate crystals on calcium hydroxide-sprayed and inoculated leaves. These crystals were morphologically different from amorphous calcium hydroxide aggregates observed in the corresponding controls.

## DISCUSSION

The application of calcium hydroxide resulted in complete inhibition of leaf spot development both on detached and undetached leaves. The decreases in lesion numbers with increases in calcium hydroxide spray concentrations pointed to the importance of using the correct spray concentration in disease control.

Our previous observations (5,6) showed that the pathogen secretes oxalic acid which sequesters calcium cations from the leaf, resulting in the formation of calcium oxalate. It is conceivable that the observed calcium oxalate crystals on the calcium hydroxide-sprayed inoculated leaf surface (Fig. 1B) are a result of sequestration of calcium from calcium hydroxide in preference to that from the host. That oxalic acid readily sequesters calcium from calcium hydroxide was further ascertained by conducting experiments in vitro by incubating gemmae in the presence of calcium hydroxide in water only. This also resulted in the formation of calcium oxalate crystals (Fig. 3A,B) similar in morphology to crystals observed on calcium hydroxide-sprayed inoculated leaves (Fig. 1B). These results indicate that calcium hydroxide, when applied externally, can control the pathogen possibly by way of neutralization of oxalic acid through calcium oxalate formation and preventing sequestration of host calcium.

In summary, the results suggest that calcium hydroxide, if applied at a proper concentration, can effectively check the disease and they indicate a potential for its use in the field control of the American leaf spot of coffee. Different sources of calcium are being currently tested in the field in Costa Rica and the results obtained so far have been encouraging (E. Vargas, *personal communication*).


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