Occurrence of Magnesium Oxalate Crystals on Lesions Incited by *Mycena citricolor* on Coffee

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ABSTRACT

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Numerous rhombic crystals were observed on the lower surface of 3-wk-old lesions on coffee leaves incited by *Mycena citricolor*. Scanning electron microscopy showed that these crystals exist singly or as aggregates. The single crystals exhibited rhombic configuration, and crystal aggregates were organized as druses or crystal conglomerates. Energy-dispersive X-ray microanalysis and mass spectrometry provided evidence that the crystals were composed of magnesium oxalate. Similar rhombic crystals also were observed on the upper surface of 3-wk-old lesions, either present singly or coexisting with prismatic calcium oxalate crystals. It is postulated that magnesium oxalate crystals, which were absent in the control treatments, were formed in the infected tissue through sequestration of leaf magnesium by fungal oxalic acid. This is the first report on formation of magnesium oxalate subsequent to infection by a fungal pathogen.

Additional keywords: American leaf spot of coffee, cation sequestration, *Ojo de gallo*, *Omphalia flavida*.

The American leaf spot or *ojo de gallo*, caused by *Mycena citricolor* (Berk. & Curt.) Sacc., is an economically important disease of coffee in the Latin American countries (3,17). The main symptoms include brownish leaf spots and extensive defoliation. The pathogen also infects young twigs and berries. Recently, we demonstrated the production of oxalic acid and sequestration of calcium by this pathogen (13,15). We postulated that oxalic acid plays a key role in pathogenesis by sequestration of host calcium. In this article we describe magnesium oxalate crystals on coffee leaves infected with *M. citricolor* and interpret their

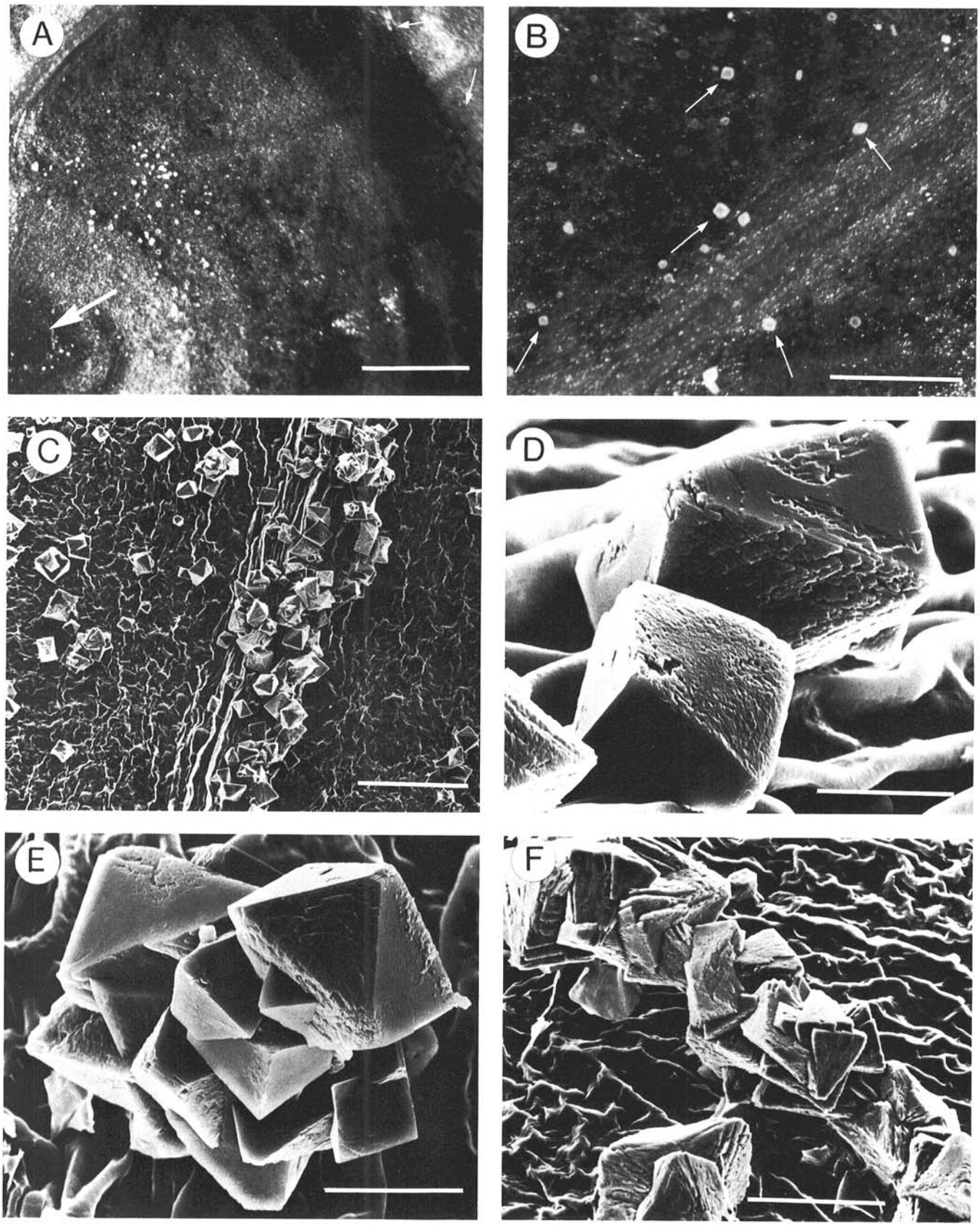

Fig. 1. Crystals and crystal aggregates of magnesium oxalate on the lower surface of the lesions on coffee leaves incited by *Mycena citricolor*. **A,** Light micrograph of lesion surface, showing single crystals. Large arrow indicates lesion center, and small arrows indicate lesion margin. Note the crystals on the lesion. Bar = 1,000 μm. **B,** View of A at higher magnification. Arrows indicate single crystals. Bar = 300 μm. **C,** Low-magnification scanning electron micrograph (SEM), showing predominantly single rhombic crystals. Many are associated with the vein. Bar = 200 μm. **D,** SEM close-up of two single rhombic crystals. Bar = 15 μm. **E,** SEM of druse aggregate. Bar = 20 μm. **F,** SEM of crystal conglomerate, showing angular delineations. Bar = 100 μm.

formation as a consequence of sequestration of leaf magnesium by fungal oxalic acid. This is apparently the first report of magnesium oxalate formation by a pathogen that produces oxalic acid.

MATERIALS AND METHODS

Culture of *M. citricolor*. A Costa Rican isolate of *M. citricolor* (TEW 2), supplied by E. Vargas, was used. It produces oxalic acid in culture and in infected tissue (13,15). The culture was maintained as described previously (13).

Coffee plants and leaf inoculations. Coffee plants (*Coffea arabica* L. 'Caturra') were grown, and detached leaves were inoculated with the pathogen as described earlier (13,19). Six leaves were used in each experiment, and the experiments were repeated four times. The interveinal areas of both halves of the upper leaf surface were scratched. Each scratched point on the right half was inoculated with three gemmae; the scratched points on the left half served as controls. The leaves were incubated in Petri dishes containing moistened sterilized vermiculite at 20 C with a 12-hr light cycle of 28 μE m^{-2} sec^{-1}. One and three weeks after inoculation, microscopic observations were made for crystal formation on upper and lower surfaces of leaves.

Scanning electron microscopy (SEM) and energy-dispersive X-ray microanalyses. Fifty single rhombic crystals were collected with forceps from the lower surface of 3-wk-old lesions. Crystals on the upper and lower surfaces of lesions were examined for their shape and for the presence of major cations. Five lesions were taken from the inoculated leaves from each experiment, and pieces of lesion tissue, with crystals present on their surfaces, were glued to SEM stubs by Scotch double-stick transparent tape and air-dried. For crystal morphology studies, the specimens were coated with gold in a sputter coater for 90 sec. Energy-dispersive X-ray microanalyses were made before the gold coating. Micrographs were taken at 20 kV in a Cambridge Stereoscan 250 scanning electron microscope equipped with a Kevex Micro-X 7000 analyzer.

Mass spectrometry. To the collected crystals (1 mg) from the lower lesion surface, 0.1 ml of concentrated hydrochloric acid in 0.3 ml of double-distilled water was added and shaken for 5 min. The solvent was evaporated in vacuo, and the residue was triturated twice in diethyl ether. The combined ethereal solution was cooled to 0 C, and excess diazomethane in ether was added and maintained at 0 C for 20 min. The ethereal solution then was washed with water, dried over anhydrous sodium sulfate, and filtered, and the solvent was evaporated. The resultant methylated residue was submitted to low-resolution electron impact mass spectrometry using an A.E.I. model MS-9 mass spectrometer. The experiment was repeated once.

RESULTS

M. citricolor incited chocolate brown necrotic lesions on coffee leaves 1 wk after inoculation (13). Three weeks after inoculation, several lesions were coalesced and formed blotches. Crystals were associated only with lesions and were absent in areas outside the lesions. They were observed only on the upper surface in 1-wk-old lesions but were present on both lesion surfaces in 3-wk-old lesions. On the lower lesion surface, the crystals were associated with veinal and interveinal tissues (Fig. 1A and B). The crystals on the upper lesion surface were more abundant and were not morphologically distinguishable from crystals on the lower lesion surface by light microscopy. The crystals were absent on either surface in scratched controls.

The crystals on the lower lesion surface were present either singly or in aggregates. The single crystals were rhombic in form, variable in size, and appeared rugged on the surface (Fig. 1C and D). The crystal aggregates existed either as organized druses (Fig. 1E) or as crystal conglomerates. The conglomerates showed irregular disposition but exhibited certain angular delineations (Fig. 1F).

On the upper surface of 1-wk-old lesions, only prismatic crystals (Fig. 2A), similar to the polyhydrate form of calcium oxalate reported previously, were present (13). In 3-wk-old lesions, however, rhombic crystals were evident on the upper surface. They were present as independent single crystals or intermixed with bipyramids (13,14) of calcium oxalate (Fig. 2B).

The X-ray emission spectra of single crystals and crystal aggregates on the lower surface of the lesion showed a distinct peak for magnesium. Magnesium was the only cation present in these crystals (Fig. 3). Similarly, the crystals that were used for mass spectrometry also showed magnesium only. However, crystals on the upper surface of 1-wk-old lesions contained only calcium (13). The prismatic crystals on the upper surface of 3-wk-old lesions contained calcium, and the rhombic crystals contained magnesium.

The mass spectrum of crystals from the lower surface of the lesion had the molecular ion peak at 118, indicative of dimethyl oxalate ($C_4H_6O_4$), and a base peak at 59, indicating $C_2H_3O_2$ (Fig. 4). These results provided evidence for the presence of oxalic acid in the crystals. This, in conjunction with X-ray microanalyses, showed that the crystals were composed of magnesium oxalate.

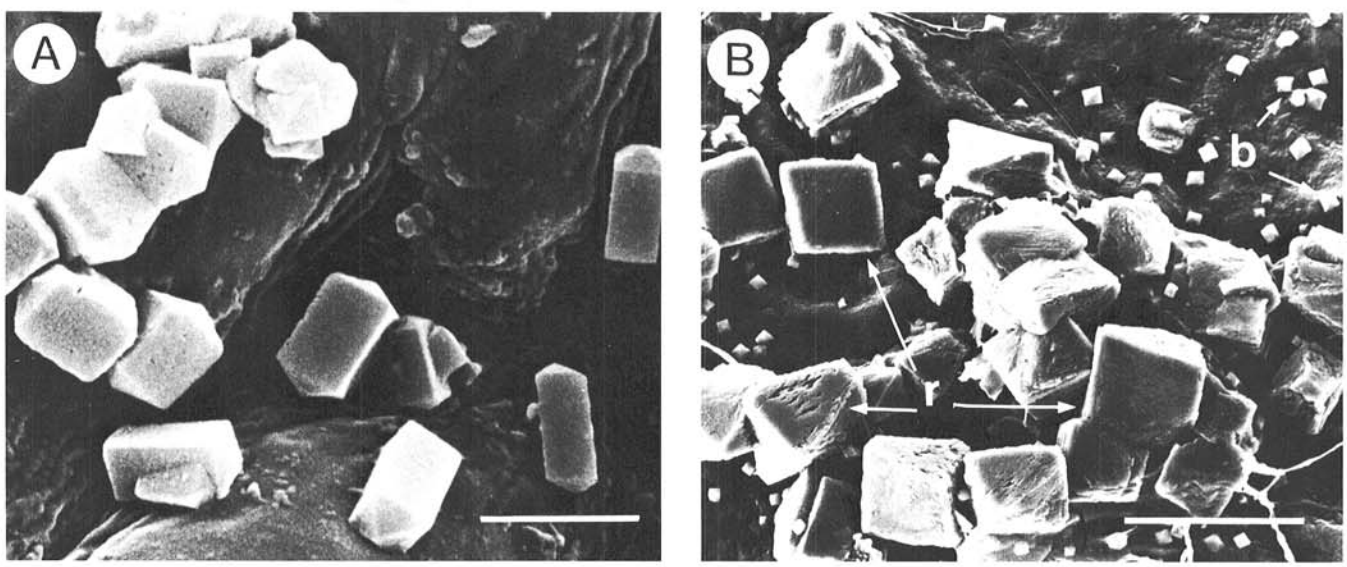

Fig. 2. Scanning electron micrographs of crystals of calcium oxalate on the upper surface of lesions on coffee leaves incited by *Mycena citricolor*. **A,** Prismatic crystals on 1-wk-old lesions. Bar = 2 μm. **B,** Crystals on a 3-wk-old lesion. Note the coexistence of rhombic crystals (r), which contained magnesium, and bipyramids (b), which contained calcium. Bar = 50 μm.

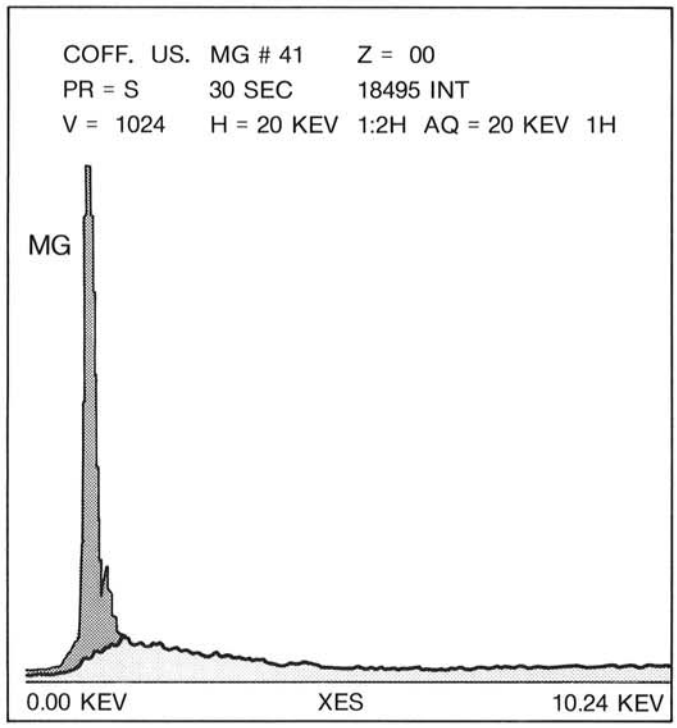

Fig. 3. Energy-dispersive X-ray spectrum of crystals from the lower surface of 3-wk-old lesions incited by *Mycena citricolor*. Note the K_α emission peak for magnesium. Base line indicates the background spectrum.

DISCUSSION

Previous studies showed that *M. citricolor* accumulated oxalic acid in infected tissue, resulting in sequestration of calcium (13,15). The demonstration of magnesium oxalate crystals in this study indicates that, in addition, the fungal oxalic acid also sequesters magnesium. Calcium and magnesium are macroelements essential to the mineral nutrition of higher plants (4,16). Whereas calcium forms insoluble salts with pectates, which make up the intercellular structural skeleton of the middle lamella, magnesium is more important intracellularly. Magnesium is present in the chlorophyll molecule and plays a pivotal role in photosynthesis (7). Furthermore, magnesium is essential for keeping the integrity of the two subunits of ribosomes, which take part in protein synthesis (2). It is an activator of many enzymes that take part in carbohydrate metabolism and ATP-mediated reactions (4). It is therefore possible that sequestration of magnesium by oxalic acid may have a unique significance in the deterioration of chloroplasts and ribosomes in the infected cell.

Very little is known about the occurrence and crystallization of magnesium oxalate in higher plants (11). In this study, magnesium oxalate crystals were observed in 3-wk-old lesions only. They were not evident in 1-wk-old lesions, although abundant calcium oxalate crystals were present in them. Calcium is more abundant than magnesium in the plant tissue (16). Furthermore, calcium oxalate is less soluble in aqueous solutions, whereas magnesium oxalate is more soluble (0.3–0.4 g/L) and requires specific conditions for crystallization, such as, ionic equilibrium, super saturation, and slow evaporation of the medium. Because of these properties, magnesium oxalate precipitates at a slower rate than calcium oxalate (6,9,10). These facts suggest a sequential sequestration and/or crystallization of the two cations in the infected tissue, calcium being the first to be precipitated. Further precipitation studies with pure cultures in media, with inclusion of the two cations, could prove these assumptions. It is not clear why magnesium oxalate crystals were present in a pure form on the lower surface but not on the upper surface. Perhaps, differences in surface characteristics on the two leaf surfaces could affect evaporation rate, thereby influencing crystal formation.

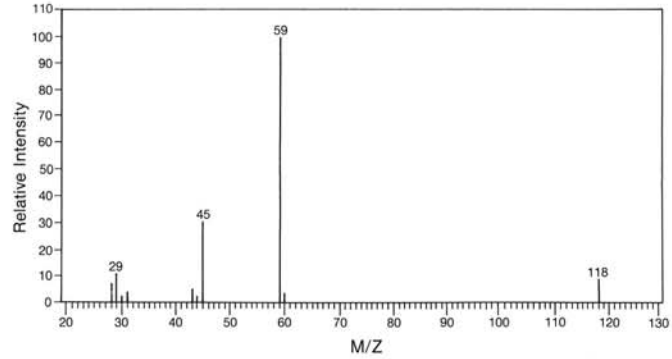

Fig. 4. A low-resolution mass spectrum of crystals obtained from the lower surface of lesions on coffee leaves incited by *Mycena citricolor*. The crystals were methylated to form dimethyl oxalate with a mass-to-charge ratio (M/Z) of 118.

The hydration state of the observed leaf magnesium crystals is not clear. However, since it is known that magnesium oxalate precipitates from aqueous solutions as a dihydrate in rhombic form (6), the lesion crystals also may be considered a dihydrate.

This is apparently the first report on production of magnesium oxalate crystals subsequent to infection by a plant pathogen. Also, an oxalate other than that of calcium, induced by an oxalic-acid-producing pathogen, was shown. It would be interesting to know whether magnesium oxalate can be formed during the pathogenesis by other oxalic-acid-producing pathogens (1,5,8,12) and whether fungal oxalic acid complexes with other cations such as iron, manganese, and potassium in the infected host. It will be also of interest to study the role of magnesium compounds in disease control, because calcium compounds are known to control the American leaf spot of coffee by neutralizing oxalic acid (14,18).

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