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Trichoderma koningii as a biomineralizing fungous agent of calcium oxalate crystals in typical Argiudolls of the Los Padres Lake natural reserve (Buenos Aires, Argentina)

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Abstract

The aim of the present study, performed on typical Argiudolls in a natural reserve with little or no anthropic impact, was to characterize the fungous biomineralizing process of calcium oxalate crystals in organic horizons of the soil. The chosen sites possessed different plant cover, identified as acacia woods and grassy meadows with particular micro environmental conditions that have differing effects in the process of biomineralization. The contribution of the plant material in the soil is a key factor since 1) it generates the particular composition of the organic horizons, 2) it determines the nature of decomposing organisms, and 3) it affects the presence, composition and development of biominerals. According to the results obtained, the acacia woods prove to be a site comparatively more favorable to the fungous biomineralizing process. This makes itself manifest in the greater abundance and development of crystals in the organic horizons of the soil, resulting in whewellite ($CaC_2O_4 \cdot H_2O$) and weddellite ($CaC_2O_4 \cdot (2+x)\ H_2O$) regarding biomineral species developed, the latter being the major component. The observation of both species of biominerals is noteworthy since it represents the first cited in the country. The isolated fungous organisms were *Trichoderma koningii,* and *Absidia corymbifera. T. koningii* was identified as the most active biomineralizing organism thus constituting the first reference to indicate this species as a biomineral producing agent.

Key words: whewellita – weddellita – biomineralization – Argiudoll – *Trichoderma koningii*

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Introduction

The process of biomineralization, by which organisms generate crystalline formations of diverse composition, is a widespread phenomenon in nature. In the particular case of calcium oxalate, its presence in geological environments has been considered extremely unusual, even though its appearance proves quite common in animal and plant tissue (Graustein *et al.* 1977), as well as in fungi and lichens (Jones *et al.* 1981).

According to its composition, two different species of calcium oxalate biominerals can be distinguished: whewellite ($CaC_2O_4 \cdot H_2O$) and weddellite, from its dehydrated forms ($CaC_2O_4 \cdot 2H_2O$) to the polyhydrated ($CaC_2O_4 \cdot (2+x)\ H_2O$) (Franceschi and Horner 1980).

Regarding its presence in the Fungi kingdom, from the very first identification made by Schmidt in 1847 (Horner *et al.* 1983), the observation and study of both mineral species have been the motivation behind numerous research projects. These studies include the presence of crystals both in reproductive structures and in vegetative hyphae (Horner *et al.* 1983; Whitney and Arnott 1986 a, b, 1987; Larsson 1994).

The fungous biomineralization process can occur intro and/or extracellularly. The first form is associated with the metabolism of vegetative growth and degrading of the organic resource while the extracellular biomineralization is related to the activities of mycorrhizal species. These species accumulate Ca^{++} preferably in the walls of the hyphae according to the activity of excreted

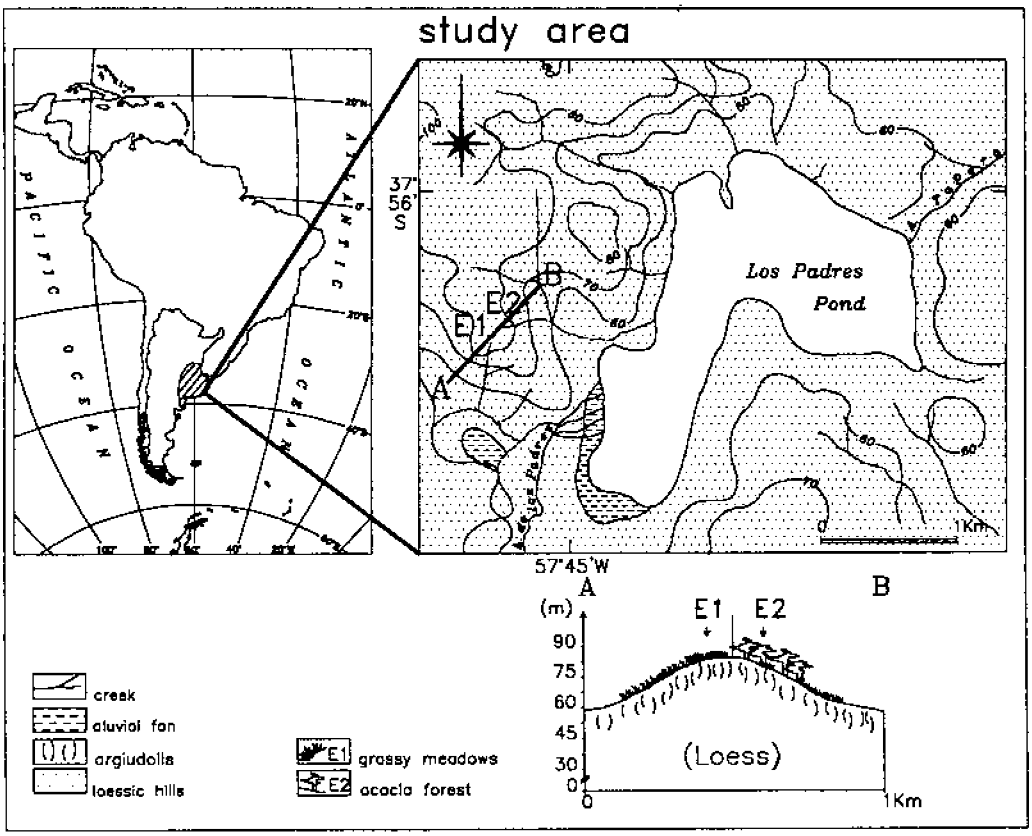


Fig. 1. Study area. Los Padres Lake Natural Reserve with differing ground vegetation: grassy meadows (E1) and acacia woods (E2).

oxalic acid which reacts with available calcium in the environment to form calcium oxalate salts (Verrecchia *et al.* 1993).

Among the species which produce calcium oxalate crystals are the various Basidiomycetes, such as: *Hysterangium crassum* (Cromack *et al.* 1979), *Paxillus involutus* (Lapeyrie *et al.* 1990) and *Hebeloma crustuliniforme* (Froidevaux and Kälin 1981), as well as Ascomycetes: *Dasyscypha capitata* (Horner *et al.* 1983) and Mucorales: *Mucor mucedo, M. phimbesis, Cunninghamella echinulata* (Franceschi and Horner 1980) and *Gilbertella persicaria* (Whitney and Arnott 1986a).

Beyond the biogenic source and mechanisms considered, the resulting biominerals in the edaphic environment could constitute, from the point of view of plant nutrition, an important reservoir for calcium within the soil.

The aim of this paper is to: 1) characterize the fungous biomineralization of the calcium oxalate crystals at the organic horizons of the soil in terms of development and crystalline habit, and 2) identify the biogenic source/s.

Materials and methods

Area of study. The study was performed on typical Argiudoll soils, developed on the geomorphological unit perirange eolian hills of the Los Padres Lake Natural Reserve which possesses differing ground vegetation: a) acacia woods and b) grassy meadows (Map, Fig. 1).

The climate of this area is mesothermic and subhumid with zero water deficiency. Average annual precipitation is 809 mm and average annual temperature is 13.7 °C. Soil temperature is of the mesic type and the system of soil moisture is udic (Soil Survey Staff 1975).

The hills of relative heights of 50 and 70 m a.s.l. show a predominance of superficial wash, which together with the dense ground vegetation in both sites create a predominance of sheet runoff (Osterrieth *et al.* 1996).

The mineral fraction of the typical Argiudoll is of a silt loam texture and does not possess calcium carbonates in the solum; levels of calcretes only appear at a depth of below 2.5 m. The mineralogical analysis of the fine fraction indicates enrichment in clay smectites and interstratified irregular smectite-illite on the illuvial

Table 1a. Morphology and physicochemical characteristics of Acacia woods.

Horizon	Depth (cm)	Wet Color	Structure	Cutans	Texture	pH	Roots
Oi	0–3	–	–	–	–	–/5,95	–
Oe	3–7,5	–	–	–	–	5,44/5,35	–
Oa	7,5–9	–	–	–	–	5,51/5,20	++(a)
A	9–45	10YR 2/1	Granular medium moderate	–	SL	5,90/6,21	+++(a)
B1	45–50	10YR 3/2	Blocks medium moderate	Humic-clay +	SL	n.d.	+
B2t	50–70	10YR 3/2	Medium prismatic to moderate/strong	Humic-clay +++	SL	n.d.	+
B3t	70–114	10YR 4/3	Medium prismatic to moderate/weak	Humic-clay ++	SL	n.d.	+
C	114+	10YR 5,5/4	Massive	–	SSL	n.d.	++

References: + = scarce, ++ = abundant, +++ = very abundant, (a) = no acacia roots. SL = silt loam, SSL = sandy silt loam, n.d. = no date.

Table 1b. Morphology and physicochemical characteristics of grassy meadows.

Horizon	Depth (cm)	Wet Color	Structure	Cutans	Texture	pH	Roots
Oi	0–3	–	–	–	–	–/6,30	–
A	3–42	10YR 2/1	Granular medium moderate	–	SL	6,10/6,35	+++
B1	42–50	10YR 3/2	Blocks medium moderate	Humic-clay +	SL	n.d.	+
B2t	50–70	10YR 3/2	Medium prismatic to moderate/strong	Humic-clay +++	SL	n.d.	+
B3t	70–114	10YR 4/3	Medium prismatic to moderate/weak	Humic-clay ++	SL	n.d.	+
C	114+	10YR5,5/4	Massive	–	SSL	n.d.	++

References: + = scarce, ++ = abundant, +++ = very abundant. SL = silt loam, SLA = sandy silt loam, n.d. = no date.

horizons (Bt). The content of organic material and nitrogen are high in both locations, as are the values in their capacity for cationic exchange, intervening with decreasing importance ions Ca^{++} and K^{+}, Mg^{++}, Na^{+} (Osterrieth and Maggi 1996). The soil profiles of both sites possess physicochemical characteristics and morphology similarities (Table 1, a and b).

Methodology. The material studied, coming from different organic horizons of the soil, was collected in successive samplings performed during the period of March 1995 to April 1998. In every instance the randomly collected samples were divided into five sub-samples and were conserved at a low temperature (5 °C) until being processed. Five replicas were utilized, all coming from each sample of the material collected, in order to locate a qualitative determination of the amount of fungous covering through the use of a binocular magnifying glass. Afterwards, from the same fungous mats, four fractions were isolated, each for a specific use: 1) culture in a solid medium, and 2) observation under: a) a light microscope (Bausch and Lomb), b) a petrographic microscope (Carl Zeiss, Jena), in samples with a ranking of 3 x 3 mm^2 to 10 x 10 mm^2, and c) a scanning electrone microscope (SEM) (Jeol T-100 and Hitachi). For the analysis of the crystals, semiquantitative studies were employed by means of conventional EDAX and EDAX without Be window operated between 15 and 25 kV in order to measure the presence of carbon and oxygen elements in addition to calcium. Computerized images were utilized to measure the dimensions of the crystals.

The presence of calcium ions in the study sites was expressed as percentile content of Ca^{++} with respect to the total sample analyzed (grams of calcium per each 100 grams of dry weight of the sample).

The fungous isolation obtained from the organic debris analyzed, was performed on plates from direct hyphal isolation in malt extract agar enriched with peptone and dextrose, and with the addition of an antibiotic solution (chloramphenicol 30 mg/liter) (Kendrick and Parkinson 1990). The plates were incubated at room temperature (22–25 °C) with daily observation during fifteen days, to make sure that any fungous growth that might have occurred would not originate from the plated hyphae.

Results

Description of organic horizons.

The acacia woods have an abundant accumulation of plant material, mainly leaf debris, where one can observe whitish mycelial mats developing above its surface. On the Oe horizon, we observed a greater amount of fungous covering from the plant source when compared to the other organic horizons in our profile.

In the grassy meadows, the organic horizon is sparsely developed, and the fungous presence is lower in relation to the other area. The calcium ion content on the Oi horizons of the acacia forest and the grassy meadows were found to be 1.96 and 1.70%, respectively.

Fungous isolation.

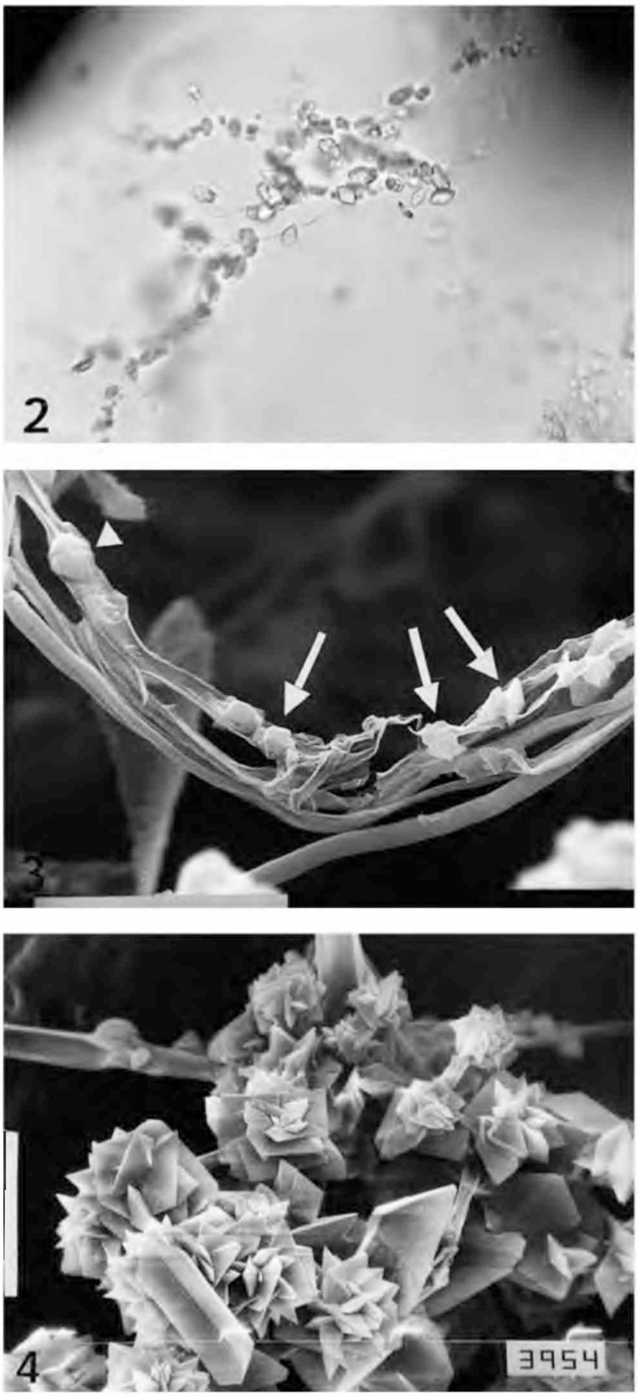
The observation of the collected material under the magnifying glasses of optic and petrographic microscopes demonstrated the presence of hyaline mycelium closely associated with crystals (Fig. 2). According to their morphology, refraction index ($\eta = 1.552$) and angle of extinction defined under a polarized light, they were revealed as calcium oxalate crystals.

As a result of the isolation, *Trichoderma koningii* and *Absidia corymbifera* were obtained. *T. koningii*, isolated within all organic horizons of woods, is cited (Rifai 1969) as a species distributed with ample environmental spectrum, including *Acacia* sp. as one of the outstanding sources of organic plant residue. Moreover, its isolation in soils associated with the meadow is quite noticeable. At the same time, *A. corymbifera*, isolated in its organic horizons, has been observed (Webster 1993) along with the rest of the members of the Mucoraceae family which develop an important activity of early colonization within organic resources.

Mineralogical and chemical characteristics of calcium oxalate crystals.

Both sites contain calcium oxalate crystals, but they were found in greater abundance in the acacia woods.

The observations made with SEM permitted to differentiate *(1)* individual crystals or primary nuclei of the bipyramidal morphology and tetragonal bipyramids with eight 1–4 µm. faces. These crystals are seen within the hyphae, similar to those described in the specific literature (Pobeguin 1943) as crystals in a letter envelope (Fig. 3); *(2)* rosettes formed by interlaced bi-



Figs. 2–4. 2. Hyaline mycelium associated with calcium oxalate crystals (light microscope: 75×). 3. Individual crystals or primary nuclei (arrows). 4. Druses and rosettes formed by interlaced bipyramidal crystals. Bar scale in Figs. 3 and 4, 10 µ (scanning electron micrograph).

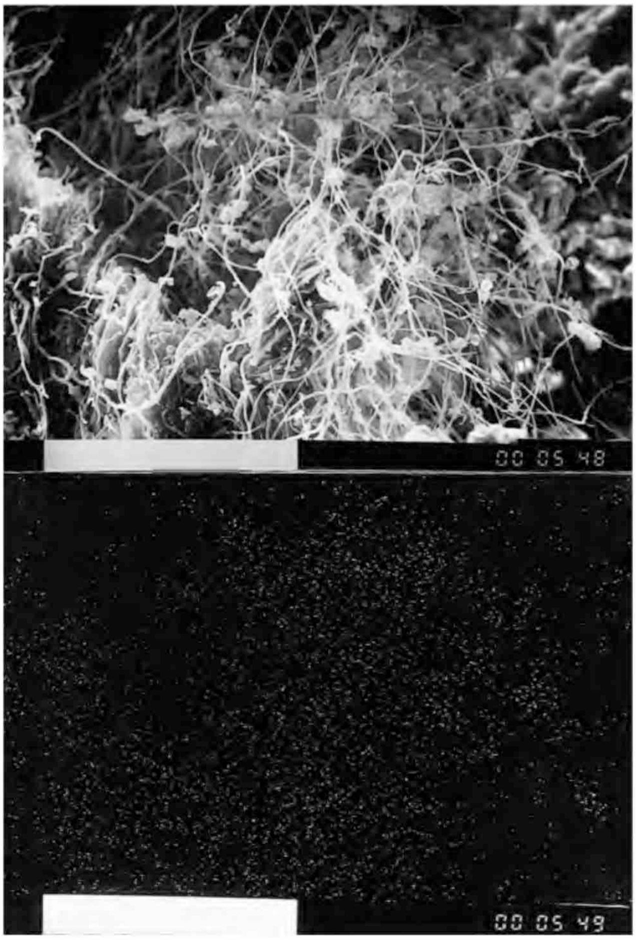


Fig. 5. Semiquantitative microanalysis on dense covering of the druses: Upper panel: Crystals forming druses associated with mycelium. Lower panel: Calcium mapping indicating presence of calcium in crystals. Bar scale, 100 μ (scanning electron micrograph).

pyramidal crystals, which become larger toward the periphery of 2–5 μm diameter, mainly positioned in a bead-like formation. Furthermore, individual rosettes were observed of 8 μm length by 5 μm width. These morphologies, which appear in all cases, maintain a close relationship with the fungous wall (Fig. 4); *(3)* druses, common morphology of irregular crystalline mass formed by aggregates of bipyramidal crystals and/or tetragonal bipyramids, closely packed, with an average size of 8–12 μm. These forms are present in great masses, and their relations with the hyphae are barely appreciable. They have been observed mainly within the horizons Oe and Oa of the acacia wood. The dense covering of the crystals forming druses can clearly be observed by means of calcium mapping (Fig. 5). Within the grassy meadows, there is a lesser quantity and smaller crystals just as there are lesser amounts of rosettes and druses.

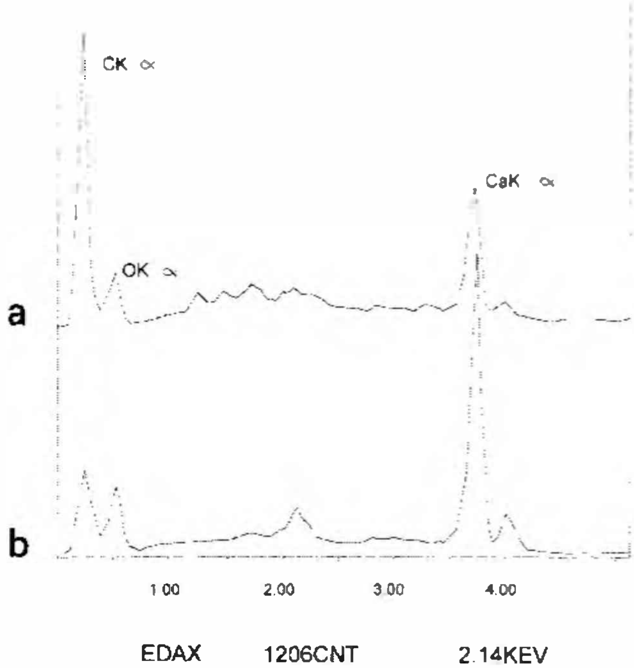


Fig. 6. Semiquantitative microanalysis by means of EDAX without Be window. Calcium oxalate: a) primary nuclei; b) druses. Axis: x = elements, y = presence.

The results of the microtests by means of EDAX without Be window confirmed that the composition of these crystals is of calcium oxalate. This analysis also demonstrated that in the primary nuclei there exist high contents of carbon and lower of calcium. In the druses this relationship becomes inverted (Fig. 6).

According to the morphological characteristics of the crystals and the existent description in the specific literature, a predominance of weddellite can be seen in all of the samples analyzed finding whewellite infrequently within the Oi horizon of the grasses.

The crystals of weddellite are apparent in all of their stages of development cited by literature (Horner *et al.* 1983), from the primary nuclei, through the intermediate stage of rosettes, and on to the stage of greatest complexity in the spatial arrangement, druses. These stages, moreover, manifested a particular degree of closeness between the fungous wall and the crystals. Observations under SEM revealed the primary nuclei situated internally regarding the fungous wall, the rosettes in close relationship with the wall but situated externally to it, and the druses with scarce connection to the fungus wall.

Discussion

The results obtained from both soil samples regarding the presence and development of calcium oxalate crystals on the organic horizons indicate that the soil from the acacia forest is the more favorable for the biomineralization process. There exists a greater composition and development of organic horizons as well as a larger associated mycelial fungous covering, and these interact to produce a greater amount of biomineral species, weddellite in particular.

The content values of the calcium ion witnin the Oi organic horizons of both soils would indicate an approximate and specific influence of this element on the soil resulting from the existent plant covering. Considering the fact that these values turned out to be similar, it could be speculated that the main difference in the influence of calcium on the soil, after comparing both sites, is principally the result of the development of each organic horizon and not the nature of the source.

The relative difference in the production of polihydrated crystals of weddellite could be explained by the elevated availability of moisture, enhanced by the composition of the B2t and B3t horizons present in the solum of both sites, which make possible a greater persistence of water in the overlying horizons (A and O).

The semiquantitative observations and analyses performed on these biominerals demonstrated a relative carbon enrichment in the primary nuclei and a close link between the fungal wall and the crystals. This would indicate that the process of biomineralization of the calcium oxalate crystals is development in an intracellular fashion. It should also be noted that various authors (Horner *et al.* 1983; Whitney and Arnott 1987) allude to the covering of fungal parietal material of the calcium oxalate crystals. The genic regulation of the process of biomineralization created by the fungus has also been mentioned (Larsson 1994).

An extracellular formation is not to be discarded, however, since fungi secrete a great quantity of oxalate into the atmosphere, which can react with the available calcium and precipitate in crystalline shapes. This reaction could occur within or above the surface of the cellular wall (Franceschi and Horner 1980). At the same time, Lapeyrie *et al.* (1990) argues the possibility the crystals, once developed, may be covered by mucilage excreted by the fungus.

Conclusions

Though fungous biomineralization occurs in both areas, the acacia woods prove to be a notably more propitious environment for the formation and development of crystals. The interrelationship between the environmental characteristics present in the sites under study and their incidence in the production of calcium oxalate biominerals can be proven in the following manner: > development of organic horizon > mycelial fungous cover > production and development of calcium oxalate crystals > presence of weddellite as biomineral species.

The determination of whewellite and weddellite represents the first record citing of the presence of these biominerals in Argentine soil, finding weddellite with three morphologies recurrent in both study sites: 1) primary nuclei, 2) rosettes, and 3) druses.

The discovery of *Trichoderma koningii* as a producer species of calcium oxalate crystals represents the first evidence of this organism as biomineralizing agent; activity developed both in acacia woods and grassy meadows. Thus, our studies have shown that *T. koningii* is a biomineralizing species, and pointing toward a hypothetical similar activity in *A. corymbifera.*

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References

Cromack Jr., K., Sollins, P., Graustein, W. C., Speidel, K., Todd, A. W., Spycher, G., Li, C. Y., Todd, R. L. (1979): Calcium oxalate accumulation and soil weathering in mats of the hypogeous fungus *Hysterangium crassum.* Soil. Biol. Biochem. **11,** 463–468.

Franceschi, V. R., Horner Jr., H. T. (1980): Calcium oxalate crystals in plants. The Botanical Review **46,** 361–427.

Froidevaux, L., Kälin, I. (1981): Accumulation d'oxalate de calcium dans les nodules du champignon mycorrhizien *Hebeloma crustuliniforme:* Importance du phénoméne pour la nutrition de l'arbre. Schweizerische Zeitschrift für Forstwesen, Journal Forestier Suisse **132,** 339–344.

Graustein, W. C., Cromack Jr., K., Sollins, P. (1977): Calcium oxalate: Occurrence in soils and effect on nutrient and geochemical cycles. Science **198,** 1252–1254.

Horner, H. T., Tiffany, L. H., Cody, A. M. (1983): Formation of calcium oxalate crystals associated with apothecia of the Discomycete *Dasyscypha capitata.* Mycologia **75,** 423–435.

Jones, D., Wilson, M., Mchardy, W. (1981): Lichen weathering of rock-forming minerals: application of scanning electron microscopy and microprobe analysis. Journal of Microscopy **124,** 95–104.

Kendrick, W. B., Parkinson, D. (1990): Soil Fungi. In: Soil Biology Guide (ed. D. L. Dindal). Wiley-Interscience Publication, USA, 49–68.

Lapeyrie, F., Picatto, C., Gerard, J., Dexheimer, J. (1990): T.E.M. study of intracellular and extracellular calcium oxalate accumulation by ectomycorrhizal fungi in pure cul-

ture or in association with *Eucalyptus* seedlings. Symbiosis **9,** 163–166.
Larsson, K. H. (1994): Poroid species in *Trechispora* and the use of calcium oxalate crystals for species identification. Mycol. Res. **98,** 1153–1172.
Osterrieth, M. L., Fernández, C., Bilat, Y., Martínez, P., Martínez, G. (1996): Interacción entre parámetros bióticos y abióticos en Argiudoles afectados por prácticas agrícolas en Laguna de los Padres, Buenos Aires. Actas XV Cong. Arg. de la Ciencia del Suelo, La Pampa **1996,** 61–62.
Osterrieth, M. L., Maggi, J. (1996): Variaciones cuali-cuantitativas de la fracción arcilla en Argiudoles afectados por prácticas agrícolas en Laguna de los Padres, Buenos Aires. VI Reunión Arg. de Sedimentología **1996,** 337–342.
Pobeguin, T. (1943): Les oxalates de calcium chez quelques angiospermes: êtude physico-chimique, formation, destin. Annales de Sciences Naturelles. Botanique, Ilème sèrie, 1–15.
Rifai, M. A. (1969): A revision of the genus *Trichoderma.* Mycological Paper Nº. 116, Commonwealth Mycological Institute, 56 pp.
Soil Survey Staff (1975): Soil Taxonomy. Agricultural Handbook 436, W.D.C., 754 pp.
Verrecchia, E. P., Dumont, J. L., Verrecchia, K. E. (1993): Role of calcium oxalate biomineralization by fungi in the formation of calcretes: a case study from Nazareth, Israel. Journal of Sedimentary Petrology **63,** 1000–1006.
Webster, J. (1993): Introduction to Fungi. Second edition, Cambridge University Press, Great Britain, 669 pp.
Whitney, K. D., Arnott, H. J. (1986a): Morphology and development of calcium oxalate deposits in *Gilbertella persicaria* (Mucorales). Mycologia **78,** 42–51.
Whitney, K. D., Arnott, H. J. (1986b): Calcium oxalate crystals and basidiocarp dehiscence in *Geastrum saccatum* (Gasteromycetes). Mycologia **78,** 649–656.
Whitney, K. D., Arnott, H. J. (1987): Calcium oxalate crystals morphology and development in *Agaricus bisporus*. Mycologia **79,** 180–187.