Biologically induced mineralization in the tree *Milicia excelsa* (Moraceae): its causes and consequences to the environment

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ABSTRACT

Iroko trees (*Milicia excelsa*) in Ivory Coast and Cameroon are unusual because of their highly biomineralized tissues, which can virtually transform the trunk into stone. Oxalic acid (C$_2$O$_4$H$_2$) and metal-oxalate play important roles in their ecosystems. In this study, the various forms of oxalate and carbonate mineralization reactions are investigated by using scanning electron microscopy and X-ray diffraction. Calcium oxalate monohydrate is associated with stem, bark and root tissues, whereas calcium oxalate dihydrate is found with wood rot fungi in soils, as well as in decaying wood. Laboratory cultures show that many soil bacteria are able to oxidize calcium oxalate rapidly, resulting in an increase in solution pH. In terms of *M. excelsa*, these transformations lead to the precipitation of calcium carbonate, not only within the wood tissue, but also within the litter and soil. We calculate that c. 500 kg of inorganic carbon is accumulated inside an 80-year-old tree, and c. 1000 kg is associated with its surrounding soil. Crucially, the fixation of atmospheric CO$_2$ during tree photosynthesis, and its ultimate transformation into calcite, potentially represents a long-term carbon sink, because inorganic carbon has a longer residence time than organic carbon. Considering that calcium oxalate biosynthesis is widespread in the plant and fungal kingdoms, the biomineralization displayed by *M. excelsa* may be an extremely common phenomena.

INTRODUCTION

The African tree *Milicia excelsa* is a complex system, in which oxalic acid and metal oxalate play important roles. Calcium oxalate in plants has been reported to have many different functions. Oxalic acid is a low-molecular-weight organic acid, which is known to play a role in the regulation of free calcium ions in cytoplasm (Robert & Rolan, 1989). In addition, calcium oxalate crystals in woody tissues are thought to render trees less palatable to termites and to act as a `combustion-retarding agent’ (Prior & Cutler, 1992). In leaves, calcium oxalate crystals provide physical protection against grazing animals. More recently, it was suggested that druse calcium oxalate crystals may help to distribute light evenly to the chloroplasts lining the radial walls and surrounding the vacuole in photosynthetic palisade cells of low-light-adapted plants (Franceschi, 2001). Calcium oxalate is particularly important because it is extremely common in plants and can represent as much as 85% of a plant’s dry weight (Robert & Roland, 1989).

No specific role has previously been proposed for oxalate in *M. excelsa*, because until now it has never been detected. Although its function remains unknown, metal-oxalate accumulation in *M. excelsa* must have important biogeochernochemical implications because oxalate minerals constitute a significant carbon source for oxalotrophic bacteria, which are numerous in both the tree and the surrounding litter. Moreover, wood-degrading fungi also influence the carbon (and some metal-cation) cycles as a result of their production of large amounts of oxalic acid and other organic acids during the decay of cellulose and lignin (Cochrane, 1958; Dutton & Evans, 1996; Gadd, 1999).

The aim of this study is to investigate the role of bacteria and fungi in carbonate biomineralization within *M. excelsa*. Biomineralization (or mineralization) is defined in this study as the processes leading to inorganic mineral deposits.

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by living matter. In *M. excelsa*, we are particularly concerned with how the biogeochemical cycles of carbon, oxalate and calcium lead to the storage of inorganic carbon in the tree. The consequences of the transformation of oxalate into carbonate have already been studied with regard to soil fauna in temperate forests (Cromack *et al.*, 1977) and in Mediterranean to semi-arid calcrite environments (Verrecchia, 1990; Verrecchia & Dumont, 1996). Until now, however, no studies have been conducted in either a tropical environment or on the consequences of such processes in terms of carbon cycling and storage.

Discussions regarding the carbon sink in forests are predominantly focused on organic carbon storage, whereas soil mineral carbon is generally neglected. However, owing to the longer residence time of carbonate minerals in soils (10^2–10^6 years compared with 10–10^4 years for soil organic matter), storage of large quantities represents a potentially more efficient sink. *Milicia excelsa* is an excellent example of an ecosystem acting as a net carbon sink because carbonate accumulation involves only atmospheric CO₂ and Ca^2+ from Ca-carbonate-free sources, i.e. calcium is not provided by the dissolution of primary (inherited) CaCO₃. Considering the global distribution and the huge number of plants able to concentrate oxalate salts (Horner & Wagner, 1995), this potential source of carbon trapping should be clearly identified.

**GEOLOGICAL SETTINGS**

Samples were collected in Cameroon and Ivory Coast from trees having about the same diameter at a height of 1.20 m from the ground. In Cameroon, two sites were investigated: the first is situated 10 km west of Lolodorf (10°47'E, 3°13'N) and the second is 15 km north of Yaounde, in the Leboudy region (11°30'E, 4°07'N). At the first site, an evergreen forest grows on a yellowish brown lateritic (i.e. ferralitic) soil >3 m total precipitation 3000–5000 mm yr⁻¹ with two wet seasons (April–July and September–December), a forest. The climate at both Cameroon sites is equatorial with ferralitic soil (<1 m), covered by a semideciduous tropical forest. The climate at this site is a semideciduous tropical forest bordering a cleared forest (Ministère du plan, Côte d’Ivoire, 2001). The mean annual temperature in Ivory Coast is 26.1 °C. *M. excelsa* is commonly logged in Ivorian forests.

**MATERIALS AND METHODS**

*Milicia excelsa* wood samples were tested for the presence of carbonate with 1 N hydrochloric acid. Wood, fractured bark and root samples were fixed with osmium tetroxide vapour and freeze-dried. Some of the samples were gold-coated, and then examined using Phillips XL20 and XL30 scanning electron microscopes with a 10 kV accelerating voltage to observe crystals formed in tissues and associated with wood-degrading fungi. Other parts of the samples were embedded in resin and prepared as thin sections (30 µm), and the remaining part of bark samples was crushed and analysed by X-ray diffraction (XRD) with a Scinagat diffractometer.

The occurrence of oxalate-degrading bacteria in soils was monitored using soil inoculum in a 15 mL DSM 81 medium (Na₂HPO₄·12H₂O 9.0 g L⁻¹, KH₂PO₄ 1.5 g L⁻¹, NH₄Cl 1.0 g L⁻¹, MgSO₄·7H₂O 0.2 g L⁻¹, ferric ammoniacal citrate 0.005 g L⁻¹, CaCl₂ 0.01 g L⁻¹, ZnSO₄·7H₂O 50 µg L⁻¹, MnCl₂·4H₂O 15 µg L⁻¹, H₂BO₃ 150 µg L⁻¹, CoCl₂·6H₂O 100 µg L⁻¹, CuCl₂·2H₂O 50 µg L⁻¹, NiCl₂·6H₂O 10 µg L⁻¹, NaMoO₄·2H₂O 15 µg L⁻¹) also containing 4.0 g L⁻¹ potassium oxalate. After the medium turned cloudy, 500 µL of the enriched culture was transferred to 15 mL of the same fresh medium. After the second enrichment, 10 µL of the culture was plated onto a solid DSM 81 medium with a sterile plastic inoculating loop (Sigma, Buchs, Switzerland). Calcium oxalate was added to this medium. The oxalate consumption by the isolated strains was controlled by observing the calcium–oxalate 4 g L⁻¹ dissolution on the DSM 81 medium as described by Tamer & Aragno (1980).

In order to monitor oxalate consumption in soils associated with *M. excelsa*, two common oxalate-degrading bacteria, *Ralstonia eturopa* (DSM 428, ATCC 17699) and *Xanthobacter autotrophicus* (DSM 432, ATCC 35674; Tamer & Aragno, 1980), were inoculated into soil extract media. One to one ratio soil extracts (1 kg soil per 1 L of water) were prepared according to the method described by Parraga *et al.* (1998). Major anions and cations of this extract were analysed by liquid chromatography using a Dionex chromatograph with a conductivity detector. Columns used for anions and cations were Dionex IonPac™ CS12A (4 mm x 250 mm) and Dionex IonPac™ CS12A (4 mm x 250 mm), respectively. Ionic composition of the soil extract was as follows: Na⁺ 13 mg L⁻¹, NH₄⁺ 6 mg L⁻¹, K⁺ 23 mg L⁻¹, Mg²⁺ 10 mg L⁻¹, Ca²⁺ 45 mg L⁻¹, Cl⁻ 18 mg L⁻¹, NO₃⁻ 3 mg L⁻¹, NO₂⁻ 150 mg L⁻¹, SO₄²⁻ 32 mg L⁻¹, and PO₄³⁻ 21 mg L⁻¹. The first medium was obtained by adding 15 g L⁻¹ agar to these extracts. A second type of medium was made by adding 4 g L⁻¹ calcium oxalate. Crystals produced on the different media were collected with tweezers, gold coated and observed with a Phillips XL30 SEM.
RESULTS

X-ray diffraction analysis of *M. excelsa* wood and bark revealed the presence of calcium oxalate monohydrate [CaC$_2$O$_4$·H$_2$O: whewellite], calcium oxalate dihydrate [CaC$_2$O$_4$·2H$_2$O: weddellite], calcium carbonate [CaCO$_3$], calcium phosphate carbonate [Ca$_{10}$(PO$_4$)$_6$CO$_3$: podolite] and amorphous silica (Fig. 1). Scanning electron microscopy (SEM) showed the presence of euhedral whewellite crystals in the bark, as confirmed by X-ray analysis. These crystals, up to 50 µm in size, seem to be principally located near or in the conductive vessels of the wood (Fig. 2A,B). At the Ivory Coast site, large amounts of whewellite were also found forming a crust around *M. excelsa* roots (Fig. 2C,D).

On decayed or rotting wood, SEM indicated the presence of weddellite associated with the hyphae of some of the wood rot fungi (Fig. 2E,F). Monohydrate oxalate crystals were observed in stem tissues in both the Cameroon and Ivory Coast samples, whereas large amounts of monohydrate oxalate associated with roots were only found in Ivory Coast *M. excelsa*. These roots do not form rhizoconcretions, but instead they are former living mineralized tissues that are found under the outer part of the root cuticle (Fig. 2C). In the latter case, it seems likely that whewellite is mainly associated with the living plant tissues, whereas weddellite is mainly related to decaying wood resulting from fungal activity. This is consistent with observations by Arnott (1995), who noted weddellite associated with wood rot fungi.

In both living and decaying wood, a large part of carbonate mineralized tissues is easily visible when thin sections of *M. excelsa* wood are observed by optical microscopy under polarized light (Fig. 3A,B). Moreover, anhedral to subhedral calcite crystals were observed filling conductive tissues using SEM (Fig. 3C,D). In addition, calcite rhombohedra and micritic aggregates were found in sap. Some of these micritic aggregates form spheres (Fig. 3E). Other carbonate features are found in sap and in decaying wood in the form of calcified tissues, including calcified cellulose fibres (Fig. 3F) and calcified cells (Fig. 3G), in which a calcified spheroid can be seen in the vacuole ghost (Fig. 3H). There is a striking difference between the Cameroon and Ivory Coast samples. In the former only traces of carbonate are found, whereas in the latter large parts of wood are replaced by carbonate.

More than 20 different morphotypes of oxalate-degrading bacteria were found in the soils surrounding *M. excelsa* (Fig. 2B). Some of these were identified as *Streptomyces* and other Actinomycetes, Pseudomonads and putative *Variovorax* (Sahin, 2003). All isolated strains quickly dissolved calcium oxalate on the DSM 81 medium, and the resulting precipitation of calcium-phosphate-carbonate was identified by XRD, optical and electron microscopy, coupled with energy-dispersive X-ray (EDAX) analysis (Fig. 4A,B; Braissant et al., 2002). For example, a rapid consumption of oxalate has been observed during the growth of a *R. eutropha* culture in a DSM 81 medium. After 48 h, oxalate is no longer present in the medium (Fig. 5). In addition, rapid dissolution of calcium oxalate was observed on a soil-extract medium to which calcium oxalate was added. On a 1 : 1 soil extract (with or without the addition of calcium oxalate) *R. eutropha* and *X. autotrophicus* show normal growth, demonstrating that an oxalate molecule is preferred to other carbon sources present in the soil extract. As the consumption of calcium oxalate by oxalotrophic bacteria progresses, the pH of the medium increases to a point at which calcium carbonate deposition is favourable. Spheroid micritic aggregates of calcite were also found in soil extract media associated with gypsum, showing that calcium oxalate associated with *M. excelsa* is rapidly consumed and transformed into carbonate (Fig. 4C,D). The precipitation of one or the other of the two minerals, calcite or podolite (a calcium-phosphate-carbonate), is mainly related to the concentration of phosphate in the different media (Stumm & Morgan, 1981). In DSM 81, phosphate is abundant because it is used as a buffer. Finally, one fungal strain was
co-isolated with bacteria on the DSM 81 medium. This strain is able to grow on oxalate as the sole carbon source, resulting in the dissolution of calcium oxalate and production of a mixed calcium-phosphate-carbonate phase.

**DISCUSSION**

The presence of whewellite in the *M. excelsa* tissues, as well as weddellite associated with fungi, correlates well with previous studies that have documented oxalate biosynthesis and occurrence in plants and fungi (Pobeguin, 1943; Franceschi & Horner, 1980; Robert & Roland, 1989; Khan, 1995; Dutton & Evans, 1996; Gadd, 1999). In *M. excelsa*, whewellite is often found in conductive tissues and is able to encrust roots. It is likely that this form of calcium oxalate is a result of the mineral nutrition of *M. excelsa* (see Jones, 1998, for a review) or in aluminium detoxification, as suggested for bacteria by Hamel et al. (1999). The presence of whewellite and amorphous silica in *M. excelsa* bark could also be related to termite protection, as suggested for acacia trees (Prior & Cutler, 1992).

Weddellite is commonly associated with fungi in decaying wood. The role of oxalic acid in fungal pathogenicity is well documented (see Dutton & Evans, 1996, for a review). However, wood rot fungi, which are not typically associated with calcium oxalate minerals, do appear to play a role in the oxalate cycle. During their consumption of cellulose, they probably help to release euhedral whewellite crystals encased in the wood tissues.

Both whewellite and weddellite are abundantly consumed by bacteria (see Eqs 1 and 2, modified from Harder et al., 1974) and other micro-organisms because no macroscopic accumulation of calcium oxalate was observed in the soil and litter surrounding *M. excelsa*.

\[
\begin{align*}
\text{CaC}_2\text{O}_4 + n\text{H}_2\text{O} & \rightarrow \text{C}_2\text{O}_4^{2-} + \text{Ca}^{2+} + n\text{H}_2\text{O} \rightarrow \text{C}_2\text{H}_2\text{O}_4^2+ \\
& + \text{Ca}^{2+} + (n - 2)\text{H}_2\text{O} + 2\text{OH}^- \\
\end{align*}
\]

(1)

\[
\begin{align*}
1000\text{C}_2\text{H}_2\text{O}_4 + 372\text{O}_2 + 32\text{NH}_4^+ & \rightarrow 32\text{C}_4\text{H}_8\text{O}_2\text{N}^{2+} \\
& + 1872\text{CO}_2 + 936\text{H}_2\text{O}. \\
\end{align*}
\]

(2)

This is consistent with the rapid organic acid consumption observed for malate by Jones et al. (1996). Regarding *M. excelsa*, the results of bacterial growth on soil extract media indicate that such consumption is also true for oxalate. There are two
principal consequences of oxalate consumption. First, metal ions are released in soils and are probably more available for other organisms, and particularly for plant and soil animals as proposed by Cromack et al. (1977). Second, there is a pH increase owing to the transformation of oxalate into bicarbonate, because oxalic acid (pk1 = 1.25, pk2 = 4.27) is ‘stronger’ than carbonic acid (pk1 = 6.35, pk2 = 10.33). This pH increase provides the required conditions for carbonate precipitation in M. excelsa environments (Eq. 3, with degassing of carbon dioxide):

\[
\text{Ca}^{2+} + 2\text{HCO}_3^- \rightarrow \text{CaCO}_3 + \text{H}_2\text{O} + \text{CO}_2.
\]

During bacterial growth on DSM 81 and soil extract media, biologically induced changes in chemical conditions also resulted in the precipitation of calcium-phosphate-carbonate (Braissant et al., 2002), along with calcite. Therefore these experimental results seem to explain the relationship between the oxalotrophic bacteria and these minerals found in the natural environment (Fig. 1A). They might also help to explain some of the contributory factors leading to the precipitation of calcite-cemented sandstones that are observed in the tropical forested ecosystems of Ivory Coast (Carozzi, 1967; Cailleau et al.). Only gypsum has neither been detected in the tree nor in the soil, although it has been precipitated in laboratory experiments; the tropical conditions probably result in its dissolution.

Based on our results, a model for the calcification of M. excelsa is as follows (Fig. 6):
1 Whewellite forms in the tree wood and bark because *M. excelsa* produces large amounts of oxalic acid as a metabolic product, which reacts with calcium ions (see 1 in Fig. 6).

2 Weddellite forms in rotting wood because, during cellulose and lignin degradation, oxalic acid is produced in large amounts by fungi. Weddellite formation results from calcium-ion chelation from the plant cell wall (Dutton & Evans, 1996; see 2 in Fig. 6).

3 Oxalotrophic bacteria present in the soil and the tree tissues (introduced through wounds) transform oxalates (Ca-oxalate and oxalic acid) into carbonate (see 3 in Fig. 6).

4 Other minerals, such as gypsum and podolite, also form as a result of local pH changes and carbonate ion production (see 4 in Fig. 6).

5 Calcite produced in the soil by bacteria (see 3 in Fig. 6) can be partially dissolved by rain (pH = 4.7). Bicarbonate ions can be pumped by the tree leading to CaCO₃ deposition, even in undersaturated conditions as described by Wollast (1971; see 5 in Fig. 6).

The differences in carbonatation between Cameroon and Ivory Coast samples are probably due to differences in hydrological conditions. It is possible that carbonate ions produced in Cameroon are leached and diluted as a result of the higher rainfall. Therefore, the accumulation of calcium carbonate inside the soil, as well as in the tree, is less likely to occur. However, all the processes described in Fig. 6 result in the accumulation of calcium carbonate in alkalinized soils associated with *M. excelsa* in Ivory Coast. In summary, calcium carbonate is derived from the oxidation of carbon originally fixed during tree photosynthesis from atmospheric CO₂ through an oxalate step. This may constitute an important potential carbon sink.

During the last century, deforestation in Ivory Coast has led to the disappearance of 12.5 million hectares of the original rainforest. *Milicia excelsa* has been extensively logged and now it only remains in protected areas (Braissant *et al*., 2003). Nevertheless, calculations using soil and tree carbonate titrations suggest that at least 1500 kg of Cₜₐ₉ᵣₑ₉ is trapped during the life of a single *M. excelsa* tree (e. 500 kg inside the tree and e. 1000 kg in surrounding soils). This value is equivalent to a sequestration of 18.25 kgC yr⁻¹.

Typically, the forestry carbon sequestration component of the global carbon cycle is linked to soil organic matter, not to soil mineral carbon (Prentice *et al*., 2001). However, as we
have shown, the abundance of carbonate minerals within *M. excelsa* testifies to the fact that the inorganic sink is of great significance to the terrestrial carbon cycle. As mentioned above, the sustainability of a carbon pool is also an important point: time residence for organic matter and mineral carbon in soil are at least $10^{11}$–$10^{13}$ years and $10^2$–$10^6$ years, respectively (Retallack, 1990; Prentice et al., 2001). Biologically induced mineral carbon sinks are undoubtedly more efficient for carbon sequestration than soil organic matter.

**CONCLUSIONS**

*Milicia excelsa* trees in Ivory Coast and Cameroon are unusual because of their highly biomineralized roots and trunk. Moreover, around these trees, calcium carbonate accumulations occur in acidic ferralitic soils, making these soils locally alkaline. This change in pedochemical conditions is made possible by the presence of oxalate in the soil and tree, which creates favourable conditions for calcium carbonate precipitation. At least three sources of oxalate contribute to the budget: the *M. excelsa* tree, fungi and bacteria. The soil microlflora includes bacteria that oxidize oxalate into carbonate, increasing the pH, and enhancing calcium carbonate accumulation. It seems that a period of low rainfall is necessary to allow calcium carbonate accumulation in the upper soil. To summarize, three conditions are necessary for calcium carbonate accumulations in ferralitic soils: (i) a large amount of oxalate, (ii) appropriate bacteria for oxalate oxidation into carbonate and (iii) a dry season. These conditions exist in many areas of tropical Africa. Consequently, carbon storage as inert calcium carbonate in soils from atmospheric CO₂ through a Ca-oxalate step constitutes a newly identified and neglected carbon sink.
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