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Experimental calcium-oxalate crystal production and dissolution by selected wood-rot fungi

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ABSTRACT

Twenty-six species of white-rotting *Agaricomycotina* fungi (*Basidiomycota*) were screened for their ability to produce calcium-oxalate (CaOx) crystals in vitro. Most were able to produce CaOx crystals in malt agar medium in the absence of additional calcium. In the same medium enriched with Ca^{2+}, all the species produced CaOx crystals (weddellite or whewellite). Hyphae of four species (*Ganoderma lucidum, Polyporus ciliatus, Pycnoporus cinnabarinus*, and *Trametes versicolor*) were found coated with crystals (weddellite/whewellite). The production of CaOx crystals during the growth phase was confirmed by an investigation of the production kinetics for six of the species considered in the initial screening (*Pleurotus citrinopileatus, Pleurotus eryngii, Pleurotus ostreatus, P. cinnabarinus, Trametes suaveolens,* and *T. versicolor*). However, the crystals produced during the growth phase disappeared from the medium over time in four of the six species (*P. citrinopileatus, P. eryngii, P. cinnabarinus,* and *T. suaveolens*). For *P. cinnabarinus*, the disappearance of the crystals was correlated with a decrease in the total oxalate concentration measured in the medium from 0.65 μg mm^{-2} (at the maximum accumulation rate) to 0.30 μg mm^{-2}. The decrease in the CaOx concentration was correlated with a change in mycelia morphology. The oxalate dissolution capability of all the species was also tested in a medium containing calcium oxalate as the sole source of carbon (modified Schlegel medium). Three species (*Agaricus blazei*, *Pleurotus tuberregium,* and *P. ciliatus*) presented a dissolution halo around the growth zone. This study shows that CaOx crystal production is a widespread phenomenon in white-rot fungi, and that an excess of Ca^{2+} can enhance CaOx crystal production. In addition, it shows that some white-rot fungal species are capable of dissolving CaOx crystals after growth has ceased. These results highlight a diversity of responses around the production or dissolution of calcium oxalate in white-rot fungi and reveal an unexpected potential importance of fungi on the oxalate cycle in the environment.

1. Introduction

Biomineralisation is the phenomenon of crystal formation by living organisms (Lowenstam, 1981; Williams, 1984). A wide array of biologically induced minerals can be crystallized, among them calcium oxalate (CaOx), which is formed when the product of the concentrations of oxalate and calcium ions is greater than the solubility product of calcium oxalate. Many plants (Nakata, 2003) and some bacteria (Hess et al., 2008) are capable of producing CaOx. Production of CaOx has also been reported in lichenous, mycorrhizal, and litter fungi (Arnott, 1995). Natural CaOx deposits can be found in wood, soil (Graustein et al., 1977; Verrecchia, 1990; Borrelli et al., 2009), and soil litter (Arnott, 1995). Modern accumulations of CaOx can be found in urban areas, street sediments, and sewage (McAlister et al., 2000), as well as on monuments (Watchman, 1991; Pinna, 1993). Despite the fact that CaOx has multiple sources and is ubiquitous, surprisingly, it has not accumulated in the geological record (Verrecchia et al., 2006).

The in vitro formation of CaOx crystals has been reported for ectomycorrhizal fungi (Graustein et al., 1977; Tuason and Arocena, 2009), airborne fungi (Mucorales; Kolo and Claeys, 2005), and biotrophic parasitic fungi (Rio et al., 2008). Studies concerning wood-decaying fungi are also plentiful. Because of their role in timber decay in buildings, brown-rot fungi have been studied in more detail than white-rot fungi (Shimazono and Hayaishi, 1957; Takao, 1965; Green et al., 1991; Dutton and Evans, 1996; Green

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and Highley, 1997; Clausen et al., 2000; Green and Clausen, 2003; Humar et al., 2005; Hastrup et al., 2006). Recently, the production of CaOx crystals by several species of oxalate-producing fungi (including the white-rot fungus *Irpex lacteus*) has been shown in relationship to the extraction of Ca^{2+} from calcium-containing minerals (i.e., gypsum) in microcosm experiments (Schilling and Jellison, 2007).

Although the production of oxalic acid appears to be widespread in fungi, the function of fungal oxalic acid is still under study. The functions known so far have been extensively discussed (for example see Green et al., 1991; and Gadd, 1999). These include: roles in pathogenicity (Dutton and Evans, 1996); several roles in lignin degradation (Shimada et al., 1994); influence on regulation of pH and favoring wood degradation (Schilling and Jellison, 2005); weathering of mineral-containing silicates (Fomina et al., 2006); regulation of bioavailability of phosphate and sulfate (Dutton and Evans, 1996); detoxification of metals, including calcium, zinc, cobalt (Jarosz-Wilkolazka and Gadd, 2003), and copper (Munir et al., 2005); and production of energy (Munir et al., 2001).

In soil oxalate is a molecule that can connect the metabolism and activity of different types of macro and microorganisms, and thus the capability of fungi to produce CaOx suggests that they may play an important role in soil ecology (Verrecchia et al., 1990; Morris and Allen, 1994; Dutton and Evans, 1996; Verrecchia and Dumont, 1996; Gadd, 1999; Burford et al., 2003; Borrelli et al., 2009). This is clearly the case for the oxalate–carbonate pathway, a process in which oxalate produced by plants is metabolized by soil bacteria. Oxalate consumption leads to the alkalinization of the medium and the precipitation of calcite in carbonate-free acidic soils (Braissant et al., 2002; Cailleau et al., 2004; Verrecchia et al., 2006). Fungi have always been regarded as important participants in this pathway because of their ability to degrade organic matter, rendering plant-produced oxalate available for bacteria. However, their role as producers and potential consumers of oxalate has never been explored.

In this study, a spectrum of white-rot fungi was selected for their ability to produce calcium-oxalate crystals. The screening included 26 species of Agaricomycotina fungi (25 wood-rot fungi and, for comparison, the non-wood-rot *Agaricus blazei*). The organisms were grown in malt agar medium. This medium was also supplemented with calcium carbonate to assess the role of Ca^{2+}. A more detailed characterization of CaOx crystal production was carried out with a subset of species. After the end of their growth, some of the species provoked the dissolution of previously accumulated CaOx. Therefore, additional experiments were conduced to test CaOx dissolution on a mineral medium supplemented with Ca-oxalate as sole carbon source.

2. Materials and methods

2.1. Organisms

All white-rot fungi tested in this study (Table 1) are deposited in the culture collection of the Microbiology Laboratory at the University of Neuchâtel, Switzerland.

2.2. Culture media and conditions

Malt agar medium was prepared according to Nobles (1965). This medium was used for growth and production of CaOx crystals. Malt agar is an undefined medium containing a variable amount of calcium coming from the barley grain. In addition, Ca^{2+} can originate from the agar, which contains traces of this element as impurities. In the experiments with an excess of calcium, the malt agar medium was modified adding 5 g l^{-1} of $CaCO_3$. Modified Schlegel medium with calcium oxalate was prepared according to Aragno and Schlegel (1991), with omission of the $NaHCO_3$ solution and addition of 7 g l^{-1} Ca-oxalate as sole carbon source. Each strain was cultured in duplicate in petri dishes for each medium and at two different temperatures. The cultures were incubated at room temperature (21–24 °C) under light with a light/dark cycle of 12 h/12 h and under a controlled temperature (26 °C) in the dark.

Table 1
Screening of 26 species of Agaricomycotina fungi (Basidiomycota) for their capability to produce calcium oxalate (CaOx; oxalogenesis) in malt agar (MA) and malt agar supplemented with 5 g l^{-1} $CaCO_3$ (MA + $CaCO_3$). The wood degrading capability of the strains is indicated. The main type of CaOx crystal observed is also indicated.

Species	Wood degrading ability	MA[a]	MA + $CaCO_3$[a]	Crystals[c]		
				Wed	Whe	CH
Agaricus blazei	−	+	+	+	−	−
Agrocybe aegerita	+	−	+	−	+[d]	−
Fistulina hepatica	+	+	+	+	+[d]	−
Flammulina velutipes	+	+	+	−	+	−
Ganoderma lucidum	+	+	+	+	−	+
Ganoderma tsugae	+	+	+	+	−	−
Grifola frondosa	+	+	+	+	−	−
Hericium erinaceum	+	+	+	+	−	−
Laetiporus sulphureus	+	+	+	+	−	−
Laricifomes officinalis	+	+	+	+	−	−
Lentinus edodes	+	+	+	−	+	−
Lyophyllum ulmarium	+	+	+	+	−	−
Meripilus giganteus	+	+	+	+	−	−
Pholiota nameko	+	+	+	+	−	−
Pleurotus citrinopileatus[b]	+	+	+	−	+[d]	−
Pleurotus eryngii[b]	+	+	+	+	−	−
Pleurotus ostreatus[b]	+	−	+	+	−	−
Pleurotus tuberregium	+	+	+	−	+[d]	−
Polyporus ciliatus	+	+	+	+	−	+
Polyporus squamosus	+	−	+	−	+	−
Polyporus tuberaster	+	−	+	−	+	−
Pycnoporus cinnabarinus[b]	+	+	+	+	−	+
Sparassis crispa	+	+	+	+	−	−
Sparassis laminosa	+	+	+	+	−	−
Trametes suaveolens[b]	+	+	+	+	−	−
Trametes versicolor[b]	+	+	+	+	−	+

[a] (+) degrades wood or presence of CaOx crystals; (−) does not degrade wood or absence of CaOx crystals.
[b] Species selected to study the dynamics of the CaOx production.
[c] Wed, weddellite; Whe, whewellite; CH, coated hyphae; For the morphology of the crystal, see Fig. 2.
[d] Large amount of amorphous crystals.

2.3. Screening of production of CaOx crystals

The initial screening of CaOx production was performed using microscopic observation of crystal presence in 10-day-old cultures incubated in malt agar with and without $CaCO_3$. For each species, three agar samples were removed from the central to the outer edge of hyphal growth. This technique allowed investigation of the presence of CaOx in mycelia of different ages (the outer edge of growth corresponding to the youngest part of the mycelia, and the central part corresponding to the oldest). The removed samples had a surface area of 0.5 cm^2. The samples were observed under an optical microscope with a magnification of 400×.

2.4. Quantification of crystals

Six selected fungal species were initially incubated for three days in petri dishes on malt agar. From the third day and beyond, squares of about 0.5 cm^2 of media supporting the fungi were removed along the same axis (Fig. 1). Thereafter, three additional squares were removed every day in the adjacent axis, until the 13th day, when all the petri dish had been sampled. All types of

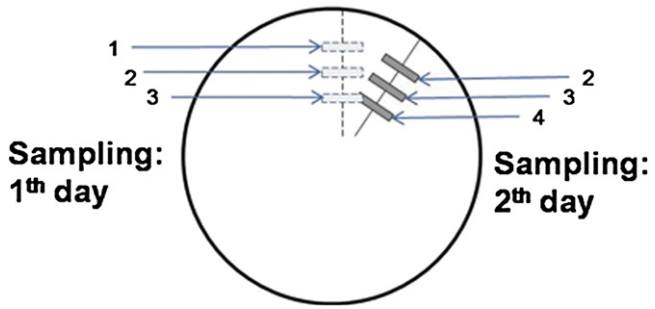

Fig. 1. Schematic representation of the sampling strategy for the quantification of crystals in fungal cultures. Age of the mycelium (in days) is shown with arrows.

calcium-oxalate crystals were counted under microscopic examination (400× magnification) in three different areas of the same sample. The macromorphology (density, color, mycelium texture) and microstructures of vegetative mycelium were observed simultaneously.

2.5. Determination of oxalic acid concentration using HPLC in cultures of Pycnoporus cinnabarinus

The HPLC method was adapted from Schilling and Jellison (2004). Additional petri dishes treated as detailed in Section 2.4 were sampled in the same way as described above for 13 days. For the sampling, fragments of 0.1 g of malt agar medium supporting the fungus were placed into 2-ml Eppendorfs. One and one half milliliters of H_2SO_4 20 mM (pH = 1.38) was added and then mixed by vortexing. Samples were incubated overnight at 20 °C with continuous shaking (45 rpm). The samples were aspirated with a syringe and then filtered through a 0.45-μm filter to remove non-dissolved particles. The filtrate was placed directly into vials that could be stored at −20 °C. The HPLC was performed by injecting 100 μl of the filtrate (three injections per sample) in an ion exchange column (300 × 7.8 mm, 10 μm, H^+ form, Benson, Reno, NV, USA) using an isocratic solution of 20 mM H_2SO_4 as eluent. The absorbance of the link C═O of the acid function was measured at 210 nm.

2.6. Screening of CaOx dissolution in Schlegel medium

The screening of CaOx dissolution by fungi was carried out by the observation of a CaOx dissolution zone around (or below) the fungal colony in the modified Schlegel medium. The plates were regularly surveyed for fungal activity for up to 60 days of incubation.

2.7. Electron microscopy

Lyophilized malt agar samples were gold-coated (10 nm) and observed using a Philips XL30 ESEM-FEG (environmental scanning electron microscope-field emission gun) coupled to an EDS (energy dispersive spectrometry) microprobe, (EDAX, USA). Peaks of calcium, carbon, and oxygen were detected with the EDS microprobe, confirming the possible weddellite and whewellite nature of the crystals.

3. Results

Twenty-six saprophytic fungi were screened for their ability to produce calcium-oxalate crystals in two different agar media (Table 1). Twenty-two of 26 fungi tested were producers of calcium-oxalate crystals on malt agar medium. In contrast, when the same medium was supplemented with $CaCO_3$, all of the 26 species were producers of calcium oxalate. The observation and identification of the main types of crystals produced (weddellite and whewellite) and the presence of crystal-coated hyphae was performed using optical microscopy. Examples of the different morphologies of the observed CaOx crystals are given in Fig. 2 for three species that simultaneously displayed all types of crystals observed in the samples. The nature of the crystals was determined by optical and electron microscopy (using crystal habits; Fig. 3A–C), and EDS analyses confirming the presence of C, O, and Ca (Fig. 3D). Variation in the crystal volume was observed. Many small crystals (size 1–2 μm) were adjacent to bigger ones (10 μm). Weddellite was found either free in the medium or associated with hyphae.

Weddellite was largely the most widespread form of CaOx formed by fungi in this study. Nonetheless, small amounts of whewellite were also observed (Table 1 and Fig. 2). For some species, the main CaOx type observed was whewellite (e.g., *Flammulina velutipes, Lentinus edodes, Polyporus squamosus,* and *Polyporus tuberaster*). Furthermore, amorphous crystals (amorphous phase) were also observed associated with *Agrocybe aegerita, Fistulina hepatica, Pleurotus citrinopileatus*, and *Pleurotus tuberregium*. For some fungi, the presence of crystal-coated hyphae was preponderant (e.g., *Ganoderma lucidum, Polyporus ciliatus, P. cinnabarinus,* and *Trametes versicolor*). Crystal coating was not homogeneous in the mycelium but localized in restricted areas.

Six of the species presented in Table 1 were selected in order to study the dynamics of the CaOx production. These species were divided into "standard" and "high" CaOx producers. The definition of standard or high CaOx producers was based on crystal counts in the visual fields observed by optical microscopy. *P. citrinopileatus* and *P. cinnabarinus* were selected as highly productive, producing a number of crystals estimated at ∼10,000 mm^{-2}. These two species were compared to the other four "standard" productive species from which ∼1000 mm^{-2} to ∼2000 mm^{-2} crystals were observed (*Pleurotus eryngii, Pleurotus ostreatus, Trametes suaveolens,* and *T. versicolor*). The deposition of CaOx crystals in the media was already observed after 24 h of incubation concomitantly to the colonization by the fungi (day 2 in Fig. 4). In both of the highly productive CaOx fungi (*P. citrinopileatus* and *P. cinnabarinus*), the number of crystals

Calcium oxalate diversity / Fungus	weddellite 	whewellite 	crystal coated hyphae 
Pycnoporus cinnabarinus	++	+	++
Polyporus ciliatus	++	+	(+)
Trametes versicolor	++	+	(+)

Fig. 2. Diversity of crystals (weddellite, whewellite, and coated hyphae) produced in vitro on malt agar by three of the wood-rot fungi analyzed. These three species were representative of the conventional dominance of weddellite over whewellite. There are no significant differences in the crystal morphologies formed by the 26 different species. However, the quantity of each crystal type varies according to the fungus: (+) occasional, + low quantity, ++ high quantity. Crystal-coated hyphae were observed principally on a malt agar + $CaCO_3$ medium.

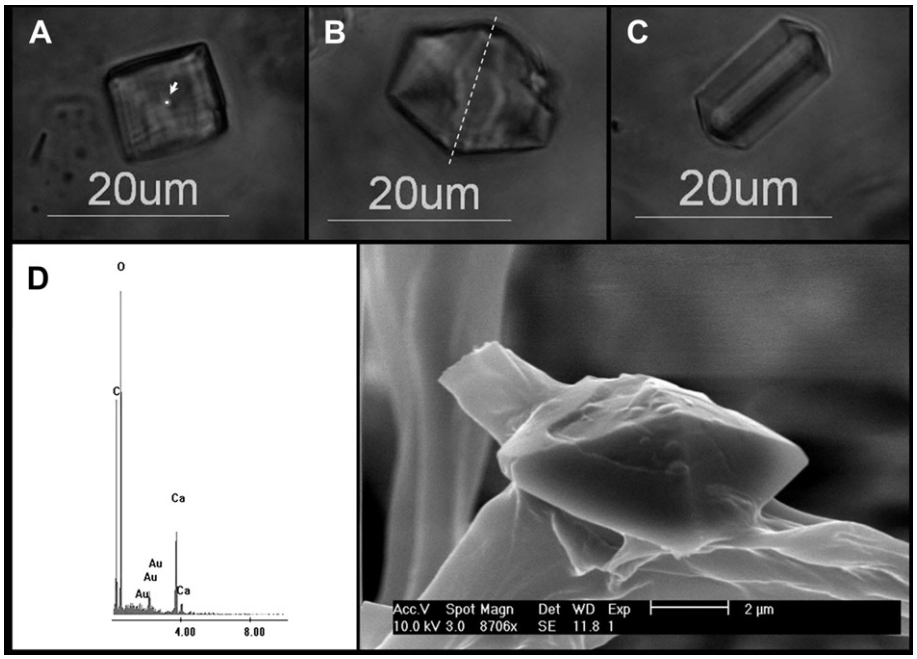

Fig. 3. Morphological (A–C) and chemical (D) characterization of weddellite. The three axes are perpendicular to each other. A: common calcium oxalate seen from the top of the *c* axis (dot and arrow); B and C: common calcium oxalate seen with its *c* axis marked with the dotted line highlighting length variation. D: Weddellite crystal ($CaC_2O_4 \cdot 2H_2O$) on the top of a *P. cinnabarinus* hyphae. On the left hand side, the respective EDAX analysis shows the presence of calcium, carbon, and oxygen. Gold is due to sample coating.

increased continuously up to the sixth day of incubation (from ~2000 mm^{-2}–~10,000 mm^{-2} to ~3000 mm^{-2}–~12,000 mm^{-2}, respectively).

For four of the six species (*P. citrinopileatus, P. eryngii, P. cinnabarinus,* and *T. suaveolens*), the disappearance of CaOx crystals formed during the initial growth phase was observed over time (e.g., from ~10,000 mm^{-2} to ~3000 mm^{-2} crystals in *P. citrinopileatus* and from ~12,000 mm^{-2} to ~3000 mm^{-2} in *P. cinnabarinus*; Fig. 4A). Considering that the disappearance of the crystals does not necessarily correlate with the degradation of oxalate but could also be due to the mobilization of Ca^{2+}, in addition to the visual observations, quantitative measurements of total oxalate concentrations were carried out for the highly productive species *P. cinnabarinus* (Fig. 5). The amount of oxalate in cultures of *P. cinnabarinus* showed an initial increase (up to 0.6514 μg mm^{-2} on the sixth day of incubation) followed by a decrease (0.3136 μg mm^{-2}; Fig. 5). The dissolution of CaOx crystals coincided with a change in the appearance of the mycelium. The colony became scattered with resistance tissues and skeletal binding hyphae. The formation of these resistant tissues was correlated with the typical absence (or very rare presence) of crystals.

The unexpected observation of the disappearance of CaOx crystals in some of the screened fungal species prompted us to try an additional screening in modified Schlegel's mineral medium amended with calcium oxalate. In this medium, only 11 out of the 26 species were able to grow (Table 2). Three species, one non-wood-rot fungus (*A. blazei)* and two white-rot fungi (*P. ciliatus* and *P. tuberregium*), were able to dissolve calcium oxalate around the mycelium (Table 2 and Fig. 6).

4. Discussion

4.1. Calcium oxalate formation

There are many reported observations on the production of calcium-oxalate crystals associated with wood-rotting fungi (Dutton et al., 1993; Schilling and Jellison, 2005; Hatakka and Hammel, 2010). The present study demonstrates that CaOx crystal production by white-rot fungi, in MA media under laboratory conditions, is also a widespread phenomenon.

Initial reports suggested that oxalic acid production in white rot was dependent on the presence of $CaCO_3$ (Takao, 1965). However in the current study, oxalic acid production was also observed in a malt agar medium not complemented with $CaCO_3$ for most of the 26 tested fungi (22 out of 26 species). However, it must be kept in mind that malt agar contains a variable amount of calcium from the barley grain and that Ca^{2+} is also found as a trace element in agar. On a medium supplemented with $CaCO_3$ (Table 1) all the fungus species studied produced crystals of calcium oxalate (CaOx). The stimulation of oxalate production by the addition of calcium is in agreement with previous hypothesis suggesting that an excess of calcium is correlated with the production of this organic acid (Bech-Andersen, 1987; Green et al., 1991; Larsen and Green, 1992; Jarosz-Wilkolazka and Gadd, 2003; Hastrup et al., 2006) In the experiments carried out with $CaCO_3$, the bioavailability of Ca^{2+} was very high and it cannot be granted that similar experiments in the presence of other Ca^{2+} sources would produce equivalent results. Therefore, similar experiments should be conducted in the presence of alternative calcium sources (e.g., minerals such as apatite, plagioclase, or alternatively $CaCl_2$ or calcium acetate), in order to test more clearly the role of Ca^{2+} in the production of oxalate by white-rot fungi.

Weddellite is the most abundant fungal crystal product according to Gadd (1999) and Arnott (1995). In addition, a coating of the hyphae with CaOx crystals has also been reported, although less frequently (Keller, 1997). In crystal-coated hyphae, the crystals seem to be initially weddellite, following a dehydration that results in the production of whewellite, with epitactic recrystallization (Verrecchia et al., 1993). Both of these, weddellite and crystal-coated hyphae, were produced in the experiments carried out in Ca-rich malt agar medium.

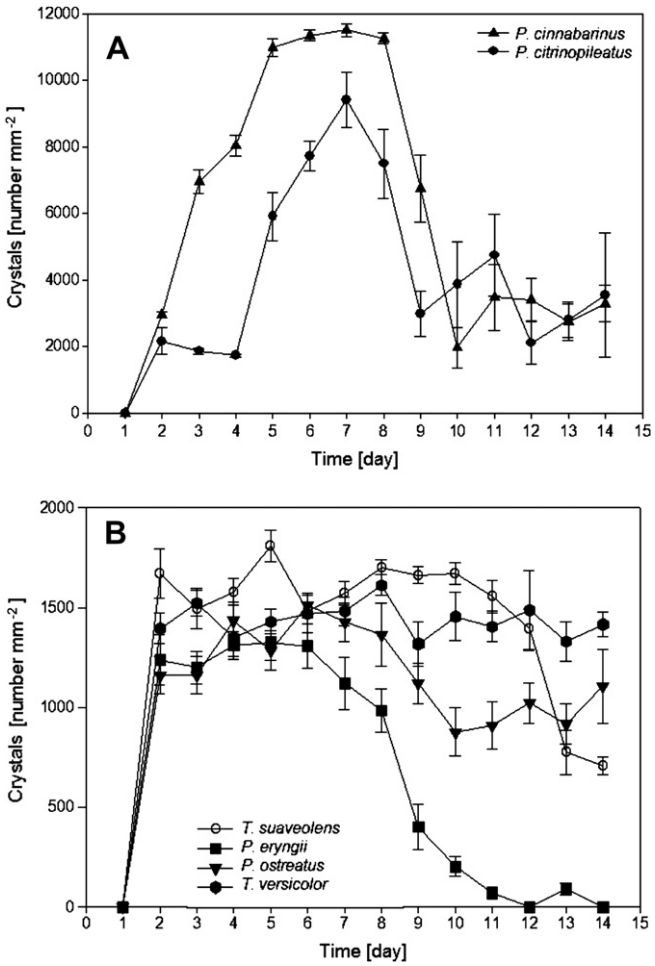

Fig. 4. Results of visual counts of calcium oxalate crystals for six fungal species growing on malt agar (number of crystals mm^{-2}). A: *P. cinnabarinus* and *P. citrinopileatus;* B: *P. eryngii, P. ostreatus, T. suaveolens, T. versicolor*. Measurements are average values of three independent measures.

4.2. Calcium oxalate disappearance

Surprisingly, when following the dynamics of CaOx crystal production, the disappearance of the crystals was observed as well

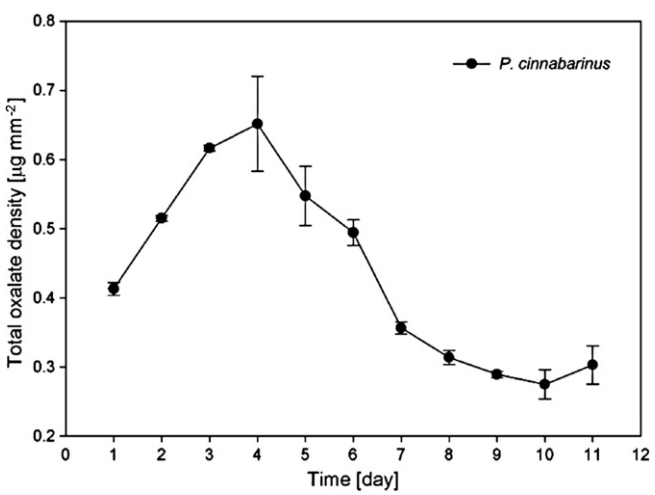

Fig. 5. Decrease in the amount of oxalic acid in medium measured by HPLC for the white-rot fungus *P. cinnabarinus* grown on malt agar. Values are an average of three independent measurements.

Table 2
Screening of calcium oxalate dissolution capability for 26 species of Agaricomycotina fungi (Basidiomycota). The capability of the species to grow on a Schlegel medium and malt agar is also recorded.

Species	Growth	Calcium oxalate dissolution on Schlegel[a]
Pleurotus tuberregium	+	+
Polyporus ciliatus	+	+
Agaricus blazei	+	+
Agrocybe aegerita	+	–
Flammulina velutipes	+	–
Ganoderma tsugae	+	–
Laetiporus sulphureus	+	–
Lyophyllum ulmarium	+	–
Pholiota nameko	+	–
Pleurotus eryngii[b]	+	–[c]
Pleurotus ostreatus[b]	+	–[d]
Ganoderma lucidum	–	–
Grifola frondosa	–	–
Hericium erinaceum	–	–
Laricifomes officinalis	–	–
Lentinus edodes	–	–
Fistulina hepatica	–	–
Meripilus giganteus	–	–
Pleurotus citrinopileatus[b]	–	–[c]
Polyporus squamosus	–	–
Polyporus tuberaster	–	–
Pycnoporus cinnabarinus[b]	–	–[c]
Sparassis crispa	–	–
Sparassis laminosa	–	–
Trametes suaveolens[b]	–	–[c]
Trametes versicolor[b]	–	–[d]

[a] (+) positive (visible CaOx dissolution), (−) negative (no visible CaOx dissolution).
[b] Species selected to study the dynamics of the CaOx production on malt agar.
[c] Positive for dissolution on malt agar.
[d] Negative for dissolution on malt agar.

(Fig. 4). The report of calcium oxalate dissolution contradicts previous reports suggesting that fungi do not appear to break down calcium oxalate due to its low solubility (Foster, 1949). Currently, Foster (1949) is still the most frequently cited author regarding the inability of fungi to degrade calcium oxalate. Recently, Tuason and Arocena (2009) showed that *Piloderma fallax* (an ectomycorhizal fungus) is capable of dissolving hyphal CaOx under limited Ca^{2+} availability, mainly as a source of Ca^{2+}. However, the mechanism of dissolution and the fate of oxalate were not investigated in detail. In the present study, two independent methods were used to prove the disappearance of CaOx crystals produced by *P. cinnabarinus* after growth. Direct counts of crystals and quantification of oxalate by HPLC both demonstrate the disappearance of oxalate from the medium. This phenomenon was accompanied by changes in the morphology of the cultures, suggesting a metabolic switch probably coinciding with the nutrient depletion. Whether the fungus assimilates the oxalate bi-anion as a carbon source or degrades it non-metabolically for other purposes remains to be tested.

Abiotic dissolution of CaOx is possible in brown-rot fungi because of a strong change in pH of the medium (pH up to 2) after few days (Ritschkoff, 1996). These fungi make a number of acids, of which oxalic acid is the most prevalent. It is a likely cause of crystal dissolution as it builds up and exceeds the concentration needed for crystal formation. By contrast, the enzymatic degradative system of white-rot fungi has an optimal pH action much higher (pH around 3–4,5, and exceptionally 5; Westermark and Ericksson, 1974; Hatakka, 1994; Swamy and Ramsay, 1999), and thus enzymatic rather than abiotic degradation could be favored. In the process of lignin degradation by white-rot fungi, degradation of oxalate by the oxalate decarboxylase (Micales, 1997; Watanabe et al., 2003) or the oxalate oxidase (Dutton and Evans, 1996) could degrade oxalate as a source of H_2O_2 that can be utilized

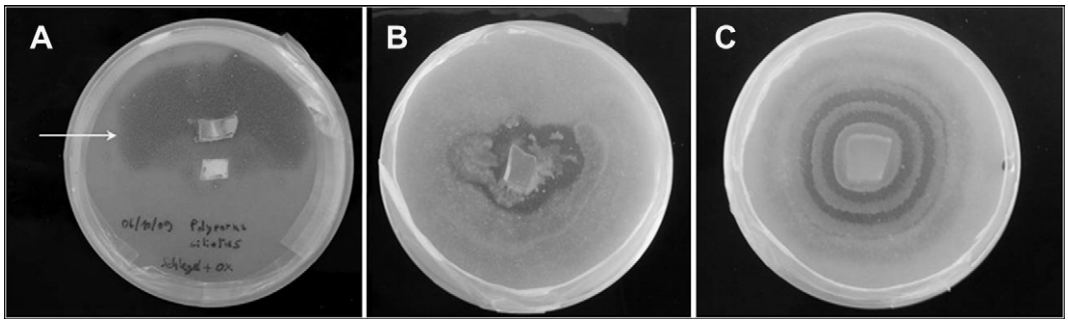

Fig. 6. Screening of calcium oxalate dissolution by a white-rot fungus on Schlegel media with calcium oxalate as sole carbon source. A: *P. ciliatus*; the mycelium has a very low biomass and is hardly seen with the naked eye. The dissolution zone is clearly visible (arrow). B: *A. blazei* showing a dissolution zone and several sparse thicker zones of mycelium biomass. C: *P. tuberregium* showing concentric dissolution zones alternating with concentric zones of mycelium biomass.

afterwards as an oxidant by lignin or manganese peroxidase (for example see Kuan and Tien, 1993). In addition, the activity of an intracellular oxalate decarboxylase has been shown in at least four species of white-rot fungi (*Dichomitus squalens, Phanerochaete sanguinea, Trametes ochracea,* and *T. versicolor*), which was also tentatively assigned to a role in the production of important intermediates in the degradation of lignin (Makela et al., 2002). However, as mentioned above, oxalate dissolution was also observed in one non-wood-rotting fungus (*A. blazei*) when the screening was carried out in a modified Schlegel's medium (Table 2), suggesting that the roles of oxalate in different species can differ.

The modified Schlegel medium was originally standardized to assess calcium–oxalate consumption by bacteria (Aragno and Schlegel, 1991), and this is, to our knowledge, the first attempt to grow fungi on it. For those rare fungal species that achieved growth (11 out of 26), the colonization generally resulted in a low biomass production, with a very thin mycelium. In addition, it was observed that three out of 11 fungi (*A. blazei*, *P. ciliatus,* and *P. tuberregium*) showed the formation of a calcium oxalate clearing (CaOx-depleted) area similar to the one reported for oxalotrophic bacteria. Although *P. citrinopileatus, P. cinnabarinus,* and *T. suaveolens* were able to dissolve crystals in malt agar medium (Fig. 4), they fail to grow on a Schlegel modified medium. Clearly, this medium is not optimal for the growth of fungi because it can lack several micro-nutrients and its initial pH (7) is probably too alkaline. The sole strain dissolving CaOx in malt agar that also grew in Schlegel was *P. eryngii*. However, in Schlegel medium, this strain did not degrade calcium oxalate, indicating that this modified medium cannot be used systematically to determine CaOx dissolution in fungi, as it is the case for bacteria.

Although, at this stage there are still several unknown facts related to the dynamics of production and consumption of calcium oxalate by fungi, the results obtained are interesting in that they highlight a potential ecological significance of fungi, which contrasts with the relatively greater importance given to bacteria in oxalate metabolism in nature (Morris and Allen, 1994). The fact that all tested white-rot fungi were positive for the production of calcium oxalate stresses their importance in the contribution to the calcium-oxalate pool and therefore their potential importance in the oxalate–carbonate pathway. Furthermore, the report of calcium oxalate dissolution by fungi supports recently published results (Tuason and Arocena, 2009). The three methods employed here show that the process of CaOx dissolution can be observed under laboratory conditions. Future studies using wood or soil will be useful to determine the true influence of fungi on oxalic acid crystal production in nature. Furthermore, pH assessments during incipient decay will shed light on the regulation of oxalic acid production.

From a larger perspective, the mechanism involved in calcium oxalate dissolution may have a number of potential applications, including bioremediation of the environment and medical treatments.

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