Translocation and incorporation of strontium carbonate derived strontium into calcium oxalate crystals by the wood decay fungus *Resinicium bicolor*[1]

Jon H. Connolly, Walter C. Shortle, and Jody Jellison

Abstract: The white-rot wood decay fungus *Resinicium bicolor* (Abertini & Schwein.: Fr.) Parmasto was studied for its ability to solubilize and translocate ions from the naturally occurring mineral strontianite. *Resinicium bicolor* colonized a soil mixture culture medium containing strontianite sand, solubilized strontium ions from this mineral phase, translocated the ions vertically, and reprecipitated the strontium into strontium-containing calcium oxalate crystals. Storage of the Sr in crystals was highest in mycelial cords and was dynamic in character. These results suggest that non-mycorrhizal saprotrophic fungi should be evaluated for their potential participation in forest nutrient cycling via biologically weathering parent material and translocating the mobilized mineral nutrients vertically within soils.

Key words: fungi, strontium, calcium oxalate, translocation, soil, minerals nutrient cycling.

Résumé : Les auteurs ont étudié le champignon de carie blanche *Resinicium bicolor* (Abertini & Schwein.: Fr.) Parmasto, quant à sa capacité à solubiliser et à transloquer les ions à partir de la strontianite, un minérai naturel. Le *R. bicolor* colonise un milieu de culture à base d'un mélange de sol contenant de la strontianite, solubilise les ions strontium à partir de cette phase minérale, transloque les ions verticalement et reprécipite le strontium dans des cristaux d'oxalate de calcium contenant du strontium. L'accumulation du Sr dans les cristaux est plus importante dans les cordons mycéliens et est de caractère dynamique. Ces résultats suggèrent que des champignons non-mycorhiziens saprophytes devraient faire l'objet d'une évaluation quant à leur capacité à participer au cyclage des éléments en milieu forestier, via l'altération biologique de la roche mère et la translocation verticale dans le sol des nutriments ainsi mobilisés.

Mots clés : champignons, strontium, oxalate de calcium, translocation, sol, cyclage des nutriments minéraux.

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Introduction

Wood decay fungi are important to the sustained productivity of forested terrestrial ecosystems (Dighton and Boddy 1989; Boddy 1991; Dighton 1995). These organisms not only transform the wood lignocellulose into humic substance precursors that become incorporated into the forest soil, they also liberate and translocate mineral nutrients through their thallus (Jennings 1990; Jellison et al. 1992; Wells and Boddy 1995; Connolly and Jellison 1997; Wells et al. 1998). The translocation of important mineral nutrients driven by the presence and distribution of coarse woody debris (CWD) could impact forest floor heterogeneity (Stark 1994; Boddy and Watkinson 1995). Such resource heterogeneity in the soil is now being recognized for its potentially important role in the functioning of natural ecosystems (Casper and Cahill 1996; Kleb and Wilson 1998).

Research on the translocation of mineral nutrients by saprotrophic fungi has primarily focused on lateral movement associated with the decomposition of organic materials (Stark 1972; Brownlee and Jennings 1982; Granlund et al. 1985; Wells et al. 1990, 1998; Wells and Boddy 1995). Although it is well known that mycorrhizae influence vertical movement of nutrients, there has been limited research on the possibility of vertical redistribution within the forest soil brought about by saprotrophic fungal interactions with lithological materials in mineral-containing soil horizons. Vertical translocation and specific biological utilization of the mineral nutrient calcium has been shown to occur in the white-rot fungus *Resinicium bicolor* (Connolly and Jellison 1995), but the precise source of the calcium was not determined. Thus, the translocated calcium could have been derived from a mixture of sources, including the decomposing wood, the soil-mixture organics, and the soil-mixture mineral phases.

Fungal interactions with mineral materials are known to occur. Much research has examined fungal interactions with mineral materials (Webley et al. 1963; Graustein, et al. 1977; Callot et al. 1985; Hirsch et al. 1995; Verrecchia and

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Dumont 1996; Jongmans et al. 1997; Sayer et al. 1997), crystalline lattices (Bech-Anderson 1987; Sayer et al. 1995; Sayer and Gadd 1997; Gharieb et al. 1998), or mineral-containing soil-like materials (Arnott 1982; Arnott and Fryar 1984; Lapeyrie et al. 1984; Connolly et al. 1996). Such interactions have been shown to occur in a broad range of rocks: carbonates (Callot et al. 1985; Verrecchia 1990), sandstones (Petersen et al. 1988; Hirsch et al. 1995; Ascaso et al. 1998), and granites (Hirsch et al. 1995; Lamas et al. 1995; Romão and Rattazzi 1996; Jongmans et al. 1997). Fungi, particularly lichen fungi, have shown the ability to grow in between the mineral aggregates of rocks (Lamas et al. 1995; Ascaso et al. 1998) as well as through individual mineral grains (Romão and Rattazzi 1996). Fungi that do grow through mineral grains can do so along cleavage planes (Lamas et al. 1995) or in directions of great resistance (Romão and Rattazzi 1996). In fact, the ability of fungi to grow through mineral-containing materials is so pervasive that it is destructive to many human structures (Hirsch et al. 1995; Ascaso et al. 1998; Gu et al. 1998). When fungi are part of mycorrhizae, growth through minerals (Jongmans et al. 1997) or the dissolution of minerals can liberate mineral nutrients and impact tree growth (Leyval 1990).

In many fungus–rock or fungus–mineral interactions the fungi chemically weather material with citric, gluconic, or oxalic acids (de la Torre et al. 1993; Dutton and Evans 1996). It has been known for more than 150 years that during interactions with mineral materials, fungi will often precipitate oxalate salt crystals (Bracconot 1825). Although oxalic acid is not always produced by fungi in mineral-rich environments (Lamas et al. 1995; Ascaso et al. 1998), in the instances that it is, it weathers mineral material very aggressively (de la Torre et al. 1993; Bech-Anderson 1987; Verrecchia and Dumont 1996). Oxalic acid is also produced by saprotrophic fungi in the absence of mineral material (Dutton and Evans 1996). Wood decay fungi, for example are well known for the ability to produce and precipitate oxalate (Arnott and Webb 1983; Eriksson et al. 1990; Dutton et al. 1993; Connolly et al. 1996). Many wood decay fungi grow in both the soil environment and in wood. Given the ability of wood decay fungi to translocate nutrients (Brownlee and Jennings 1982; Granlund et al. 1985; Wells et al. 1990, 1998; Wells and Boddy 1995), inhabit soil (Dix and Webster 1995; Jennings 1995; Connolly et al. 1996), and produce oxalic acid (Dutton and Evans 1996), it is possible that these organisms could weather mineral material below the forest floor proper and translocate mobilized nutrients vertically to more superficial layers, including decomposing wood. To test this possibility requires that weathering, translocation, and ultimate destination be demonstrated within a single experiment with a single species of wood decay fungus under culture conditions containing no agar.

Despite the substantial progress to date, there is no experimental example demonstrating all of the following within a single pure culture experiment using saprotrophic fungi: (*i*) the solubilization of ions from a lithological source, (*ii*) the translocation of the released ions in quantities and distances that cannot be explained by diffusion through the experimental apparatus or medium, (*iii*) the exact biological use and destination of the translocated element. For example, Lapeyrie et al. (1984) examined *Paxillus involutus* in agar cultures amended with mineral-containing soil material and nicely demonstrated that the fungus produced calcium oxalate crystals within the culture and amongst hyphae. Although it is likely that some of the calcium in the calcium oxalate crystals was derived from fungal interaction with the exchangeable Ca in the soil, one cannot be certain because of the presence of calcium in the agar and because of the autoclaving of the soil with the agar. In addition, translocation (metabolically driven redistribution) of calcium a significant distance was not demonstrated. Wells and Boddy (1995) elegantly showed that saprotrophic fungi can translocate phosphorus via mycelial cords throughout the forest floor. Later, Wells et al. (1998) showed that partitioning of the phosphorus in such systems is the result of differential fluxes from proximal and distal sources of phosphorus. However, in both cases, the use of the radioactive tracer analogue did not permit an examination of the release of phosphorus from soil mineral phases. Sayer and Gadd (1997) showed the incorporation of various metals into oxalate crystals after the solubilization of these metals from insoluble metal compounds. However, this biological incorporation did not occur concurrently with any significant translocation, and solubilization occurred in agar-type culture conditions rather than in a soil-like matrix.

It seems intuitive that some taxa of fungi, which grow through multiple habitats, should be capable of solubilizing ions from mineral materials, translocating these ions, and using them for specific biological functions. Testing this possibility requires a traceable element within a naturally occurring mineral lattice.

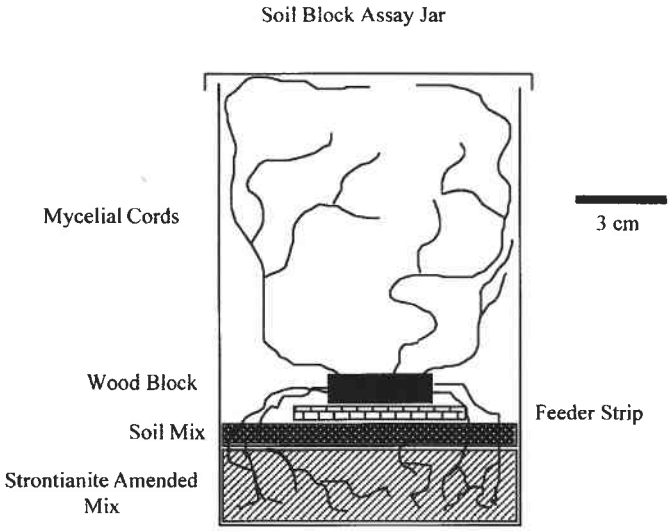
Since it is known that fungi can incorporate strontium into calcium oxalate crystals (Sayer and Gadd 1997), we used strontianite (natural $SrCO_3$) amended cultures to demonstrate the ability of the white-rot fungus *R. bicolor* to mobilize, translocate, and incorporate strontium within a single experiment.

Materials and methods

Decay microcosms

A modified American Society for Testing and Materials (ASTM) soil block assay (ASTM 1994) was used to test for the ability of *R. bicolor* to solubilize mineral phases in the soil and translocate the liberated ions away from their source (Fig. 1). A soil mixture consisting of Hyponex® brand potting soil, sphagnum peat moss, and vermiculite at a ratio of 1:1:1 by volume was used as the substrate beneath the wood blocks. The pH of the mixture was 6.8 after autoclaving. One hundred millilitres of autoclaved soil mixture was added to 460-mL capacity Ball® wide-mouth Mason jars. To one treatment group (n = 10), 10 g of strontianite (autoclaved separately from the soil mixture at 121°C for 30 min in individual vials) particles (50–200 μm in size) was aseptically mixed into the 100 mL of soil mixture. The other treatment group was not amended with strontianite ($SrCO_3$). On top of this mineral-enriched layer was deposited an additional 1 cm of 1:1:1 autoclaved soil mixture. Fifteen millilitres of sterile water was then added to the cooled soil mixture to optimize fungal growth. After the addition of the soil to all the jars, the sides of the jars were wiped clean with sterile paper towel wetted with 0.05% HCl. The pH of the soil was measured at the end of the experiment and

Fig. 1. Microcosm jar used in this experiment. Mycelial cords emanating from the wood block grew through both layers of soil and also grew up the inside surface of the jar to the rim. In all jars, crystals were produced along the entire length of the mycelial cords.



shown to be unchanged by the scant use of the acid on the sides of the glass jar.

Fungal inoculation and introduction of wood blocks

On top of the soil was placed two birch wood feeder strips (ASTM 1994). Actively growing *Resinicium bicolor* (Abertini & Schwein.: Fr.) Parmasto was aseptically added to the jars allowing the fungus to colonize the feeder strips. Once uniform growth on the strips was observed, blocks of red spruce wood (25 × 25 × 5 mm) were added on top of the feeder strips. Cultures were observed over an 8- to 10-week period and examined microscopically once every 2 weeks. When mycelial cords reached the top of the jar, the experiment was terminated.

Scanning electron microscopy and crystal identification

Mycelium from wood blocks and mycelial cords growing on the sides of the jars were prepared for scanning electron microscopy (SEM) and energy dispersive X-ray microanalysis (EDS). Samples for SEM were dried and coated with gold. Observations of calcium oxalate crystals produced by *R. bicolor* were conducted using an AMR-1000A scanning electron microscope at 5 kV and an average working distance of 7 mm. X-ray microanalysis was unavailable using the AMR-1000A and was therefore performed on samples using an Electroscan model ES-3 ESEM (ElectroScan, Wilmington, Mass.) at 15 kV with a Noran System-Voyager software computer interface. The take-off angle was 25° and the tilt was 31°. Livetime for the microanalysis was 100 s in each case. To control for the volume of probed material, each druse was probed at dead center. Preliminary X-ray microanalysis was performed on gold-coated samples with subsequent samples being carbon coated. The phase of the observed calcium oxalate crystals was determined by X-ray powder diffraction (Azaroff and Buerger 1958). A Phillips diffractometer fitted with a Supper® Debye-Scherrer powder camera (114.6 mm in diameter) was used to identify, and note any changes in the crystal lattice caused by incorporation of strontium into the crystals. Crystal-containing mycelium was rolled into a small sphere and fixed to the end of a glass fiber. The mycelium was then exposed to a collimated beam for 48 h at 30 mV and 15 mA. The X-ray film was developed and the reflection lines were measured on an illuminated film measuring device (Azaroff and Buerger 1958). The relative strength of reflection lines (interplanar d spacings) from mycelium in both strontium amended and unamended cultures were compared with each other and to a standard pattern available on file.

Table 1. A comparison of interplanar d spacings from crystal-containing mycelium with a standard diffraction pattern for calcium oxalate monohydrate.

Standard on file	Crystals from Sr-containing jars	Crystals from non-amended jars
5.93 (100)	5.98 (strong)	5.94 (strong)
3.65 (70)	3.68 (strong)	3.64 (strong)
2.97 (45)	2.99 (strong)	2.97 (strong–moderate)
2.49 (18)	2.51 (moderate)	2.53 (moderate)
2.36 (30)	2.37 (moderate)	2.32 (moderate)

Results

After 8 weeks of decomposition, the red spruce wood blocks were covered with mycelium of *R. bicolor*. Mycelial cords of *R. bicolor* ramified through the soil in the jar including the strontianite amended layer, and also grew on the inside glass surface of the Mason jars (Fig. 1). Numerous calcium oxalate crystals were observed associated with the mycelium growing on the wood blocks, and with the mycelial cords growing on the glass surfaces as previously observed (Connolly and Jellison 1995). In cultures amended with strontianite, these crystals contained strontium ions (Figs. 2*a* and 2*b*). Areas of the CaKα peak were compared with the area of the SrLα1 and SrLβ peaks combined. In all replicates, the crystals found near the top of the jar had a lower SrLα1;SrLβ/CaKα ratio in the EDS spectra than for crystals found on the wood block (Figs. 2*c* and 2*d*). Crystals found on the wood block had an average SrLα1;SrLβ/CaKα ratio of 0.5. Crystals found near the top of the jar had an average SrLα1;SrLβ/CaKα ratio of 0.025. This was true for 10 culture replicates and a minimum of 15 probe replicates per jar. The maximum distance strontium was translocated was 10 cm, from the strontianite-enriched layer to the top of the jar. This maximum distance was achieved in a minimum of five jars. Calcium oxalate crystals produced by *R. bicolor* in the jars that were not amended with strontianite did not exhibit any peaks for strontium (Figs. 2*e* and 2*f*).

Previously, Connolly and Jellison (1995) confirmed that crystals produced by *R. bicolor* are calcium oxalate monohydrate. Powder diffraction of crystals from two replicates in this experiment confirmed that result. The incorporation of strontium ions into the crystal lattice did not cause gross shifting of the interplanar d spacings or band broadening (Table 1).

Calcium oxalate crystals were particularly abundant in mycelial cords that were mining soil or growing on the surface of the jar (Fig. 3). Many of the crystal clusters (druses) had a large hole in the center (Fig.4). In some instances hyphae were observed to be traversing through these centrally located holes (Figs. 5 and 6). These may be either hyphal bore holes (H.J. Arnott, personal communication) or sites around which calcium oxalate precipitated on hyphae. Over the course of the experiment, *R. bicolor* produced

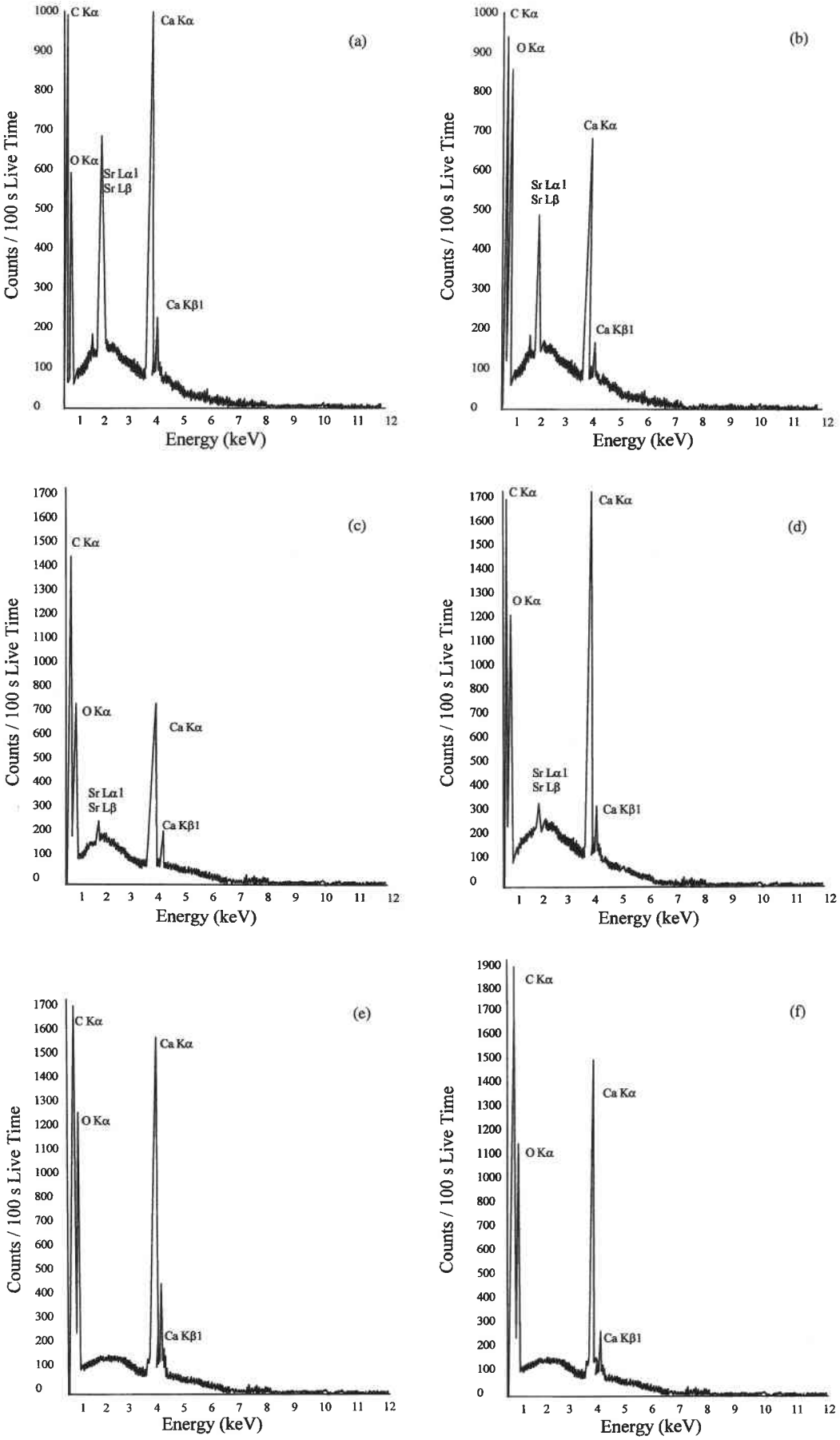
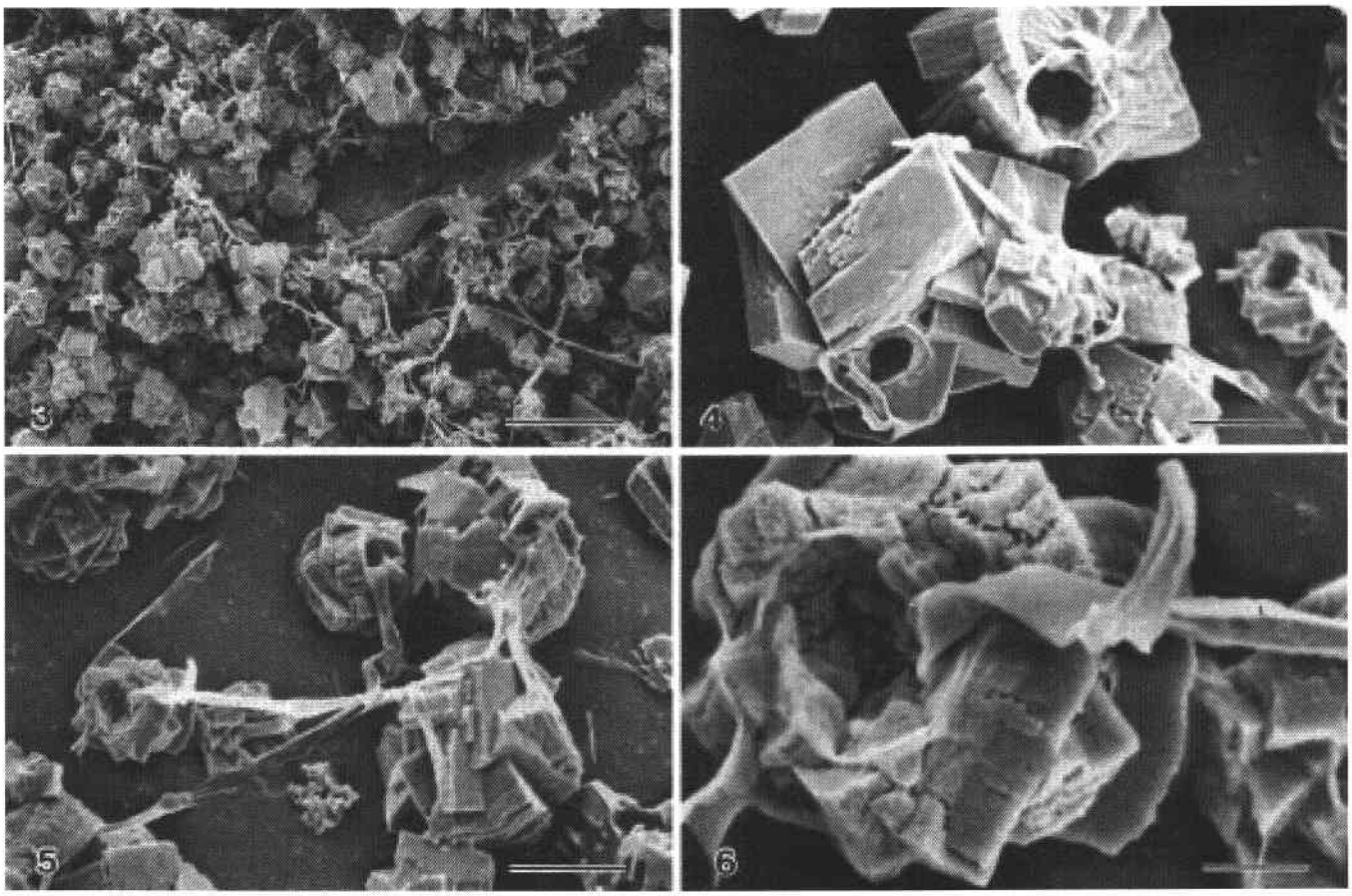


Fig. 2. Representative energy dispersive X-ray microanalysis spectra of crystal druses from the soil block cultures. Figures 2*a*–2*d* are from jars amended with strontianite. (*a*) and (*b*). Spectra of druses located in mycelium on the top of the wood block. The average SrLα1;SrLβ/CaKα ratio of peaks from the top of the wood blocks was 0.5. (*c*) and (*d*). Spectra of druses located in cords at the rim of the jar. The average SrLα1;SrLβ/CaKα ratio of peaks from the rim of the jars was 0.025. (*e*) and (*f*). Spectra of druses in mycelium located on the top of the wood block in jars that were not amended with strontianite. Note that no Sr peak was observed in any crystals precipitated in jars containing no strontianite.

Figs. 3–6. Druses of calcium oxalate monohydrate found in mycelial cords of *R. bicolor*. Fig. 3. Typical abundance of calcium oxalate crystals encrusting mycelial cords. Scale bar = 30 μm. Fig. 4. Representative druses with holes through the center. Scale bar = 5 μm. Fig. 5. Many of the "doughnut-shaped" druses still had hyphae running through them. Scale bar = 5 μm. Fig. 6. Close-up of a hypha passing through the center of a druse. Such druses may have precipitated around the hyphae, or the hyphae may have bored holes through previously existing druses. Scale bar = 1 μm.

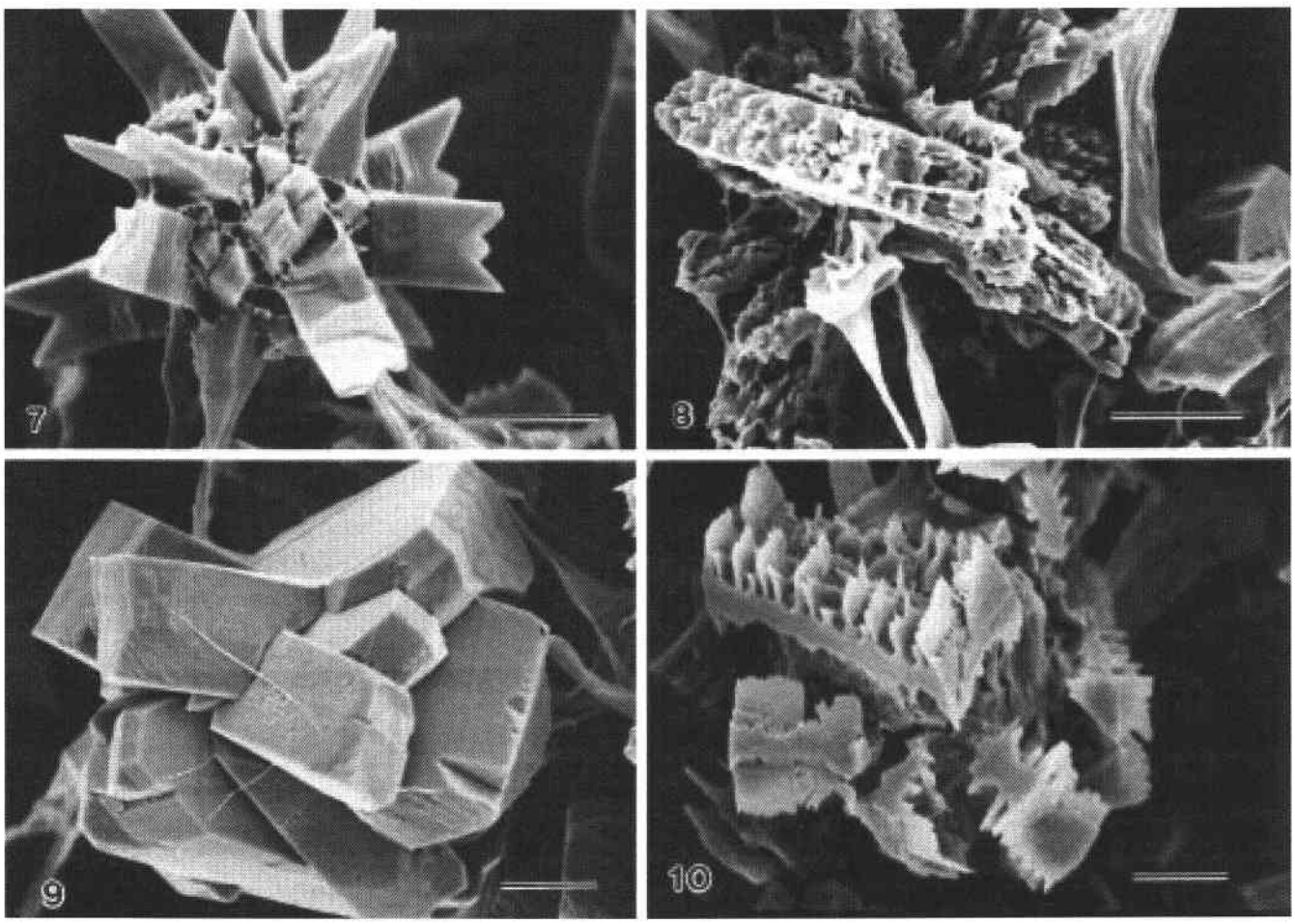


styloid druses (Fig. 7) similar to those reported earlier (Connolly and Jellison 1995). Many of these druses were corroded (Fig. 8). In addition, *R. bicolor* produced "blocky" crystals (Fig. 9) that were also subjected to corrosion (Fig. 10). Large central holes through druses were found only in the "blocky" crystal-containing clusters; none were observed in the styloid druses. Crystals from jars that were amended with strontianite did not appear to be significantly different in shape from crystals in jars that were not amended with strontianite (Figs. 11 and 12). This is similar to the findings of Sayer and Gadd (1997).

Discussion

The ability of saprotrophic fungi to solubilize and translocate mineral nutrients has been demonstrated in multiple contexts (Granlund et al. 1985; Petersen et al. 1988; Wells et al. 1990; Olsson and Jennings 1991; Jellison et al. 1992; Connolly and Jellison 1995; Wells and Boddy 1995). Most often these investigations have examined fungal interactions with solutions or organic materials. However, it is also well known that fungi can be associated with mineral materials (Webley et al. 1963; de la Torre et al. 1993; Lamas et al. 1995; Ascaso et al. 1998) bore holes through them (Romão and Rattazzi 1996; Jongmans et al. 1997) and use them to satisfy certain nutritional requirements (Bech-Anderson 1987; Wainwright et al. 1997). Research has also been conducted to examine fungal interactions with crystalline materials in the presence of other nutrient sources (Dixon-Hardy et al. 1998). Our results extend these observations by experimentally demonstrating that the saprotrophic fungus *R. bicolor* can grow through mineral-containing soil materials, solubilize ions from crystal lattices, and vertically redistribute these ions in a biogenically precipitated form.

Figs. 7–10. Corrosion of the two "morphotypes" of calcium oxalate monohydrate druses. Fig. 7. Styloid druse of calcium oxalate monohydrate. Fig. 8. Typical corrosion of styloid druse. Fig. 9. Typical "blocky" druse of calcium oxalate monohydrate. Fig. 10. Corrosion of blocky druse. Scale bars = 2.5 μm.



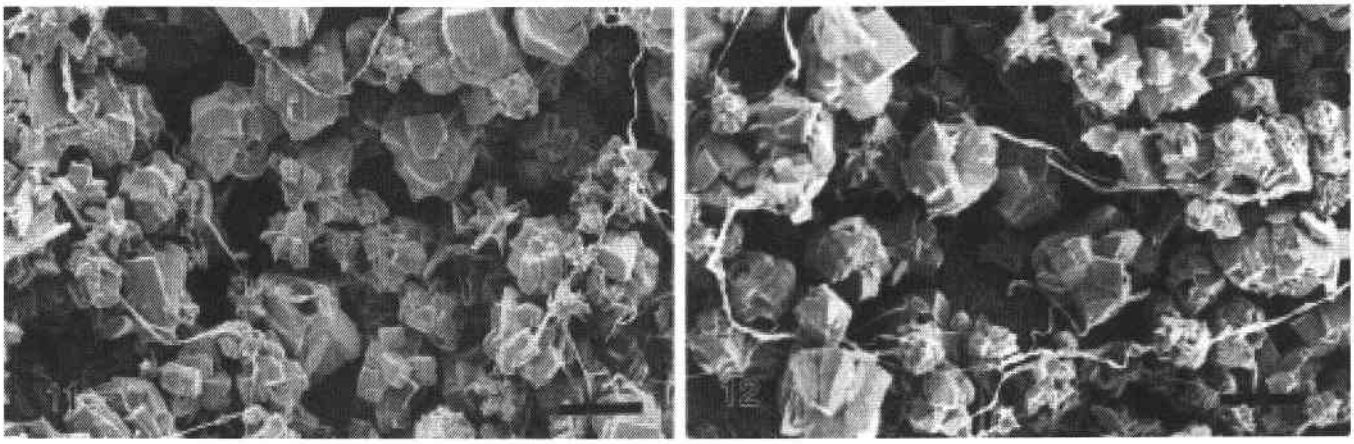
This is the first such comprehensive experimental report of which we are aware.

The appearance of strontium within the druses of *R. bicolor* only in the jars containing strontianite indicates that the strontium was derived from the mineral amendment. Autoclaving the strontianite separately from the soil mixture ensured that strontium was not released into the soil solution, thereby contaminating the experiment. Strontianite has a K_{sp} of 5.6×10^{-10}, which is less soluble than calcite ($CaCO_3$, K_{sp} 3.36×10^{-9}), but it is also far less soluble than $SrNO_3$ that has been used in other studies (Palfreyman et al. 1996; Sayer and Gadd 1997). It is possible that natural desorption of ions from the abundant stronianite surfaces added strontium to the soil solution. However, the pH of the soil was 6.8, and it is unlikely that there would be adequate desorption of strontium to incorporate into thousands of crystals without solubilization brought about by *R. bicolor*. Solubilization is most likely to be via oxalic acid production, as has been observed in so many other fungi (Callot et al. 1985; Bech-Anderson 1987; Verrecchia 1990; Sayer et al. 1995; Sayer and Gadd 1997; Gharieb et al. 1998). Strontianite, as a carbonate, is far more soluble than the silicate minerals to be found in the soils that *R. bicolor* is most likely to inhabit. Thus, it is not clear whether this fungus could extract calcium from Ca-rich silicates. However, the abundant instances of fungal dissolution of granite (Jongmans et al. 1997; Ramão and Rattazzi 1996), and the relative weatherability of Ca-containing silicates (compared with K-feldspars and quartz) suggests that they may be capable of weathering Ca-containing, silicate-based soil minerals. Our inability to obtain Sr-enriched wollastonite ($SrCaSiO_3$) prevented us from testing this directly.

The precipitation of calcium oxalate crystals in *R. bicolor* is a tightly controlled process (Nobles 1953; Connolly and Jellison 1995); thus, the inclusion of strontium into these crystals means that strontium ions were moved through the thallus via a biologically mediated path. The nature of this path is still not known. Calcium ions and their analogue, strontium, could move along hyphae via an extracellular route, or an intracellular route.

Jennings (1995) concluded that there are three possible mechanisms by which nutrients can be translocated along hyphae. One mechanism would be by diffusion along a concentration gradient that the fungus actively maintained. Precipitation of calcium and strontium could be one means of sustaining such a concentration gradient. Because hyphae are such thin, linear structures, diffusion along them could be 12 times as fast as the rate of diffusion in only one direc-

Figs. 11–12. Comparison of strontium-containing calcium oxalate crystals and calcium oxalate crystals with no incorporated strontium. Fig. 11. Representative field of strontium-containing calcium oxalate crystals. Note that both styloids and more prismatic crystals are present. Fig. 12. Representative field showing calcium oxalate crystals precipitated in jars containing no strontianite. Note that both morphotypes of druses are represented. Crystals from strontium-containing jars are grossly similar in morphology to those crystals produced in jars containing no strontium. Scale bars = 15 μm.



tion (Olsson and Jennings 1991; Jennings 1995). Such a route could be taken via the extracellular matrix of the hyphae, within the cell wall, or within the cytoplasm. A second mechanism could be via the expenditure of energy in contractile systems that move nutrients from one location to another (Jennings 1995). This would require the trafficking of discrete vesicles enriched in calcium and strontium. Lastly, there could be a genuine flow of solution within the cytoplasm not altogether unlike what occurs in the sieve tube members in vascular plants (Jennings 1995).

The association of these crystals along the hyphae of *R. bicolor* (Connolly and Jellison 1995) suggests that either oxalate and Ca, or only oxalate, is exported from the hyphae at certain loci. Perhaps these points are oxalate pumping sites (Connolly and Jellison 1995). Under either scenario, *R. bicolor* exhibits evidence of biological control over calcium and its analogue strontium in this biomineralization process. Both the mechanism and adaptive significance of this calcium translocation and precipitation is not known, but they are currently under investigation.

Wood decay fungi are well known for their ability to produce oxalic acid and precipitate calcium oxalate (Arnott and Webb 1983; Eriksson et al. 1990; Espejo and Agosin 1991; Dutton et al. 1993; Arnott 1995; Connolly and Jellison 1995). Although calcium oxalate crystals are rather insoluble, these crystals are weatherable and dynamic while the fungus is alive (Connolly and Jellison 1995). In this experiment, precipitated crystals dissolve, and either the oxalate is destroyed (Espejo and Agosin 1991; Micales 1995) or reprecipitates with calcium in new druses of calcium oxalate monohydrate. This dissolution of crystals gives rise to the observed corroded crystals. In addition, hyphae may bore holes through crystals, thus adding another element of complexity to fungal interactions with mineral materials.

The results of this study suggest that saprotrophic fungi should not be ignored for their potential role in forest nutrient cycling by weathering mineral materials in mineral-containing horizons of the soil and translocating them to surface soil layers rich in carbon-based energy. Saprotrophic fungi are most abundant in forest floor layers but can also be found in B horizons (Dix and Webster 1995). Wood decay fungi could be particularly important because of the quantity of energy stored in wood found on the forest floor in many ecosystems (e.g., Clark et al. 1998; Goodburn and Lorimer 1998). In addition, these fungi can store these nutrients in various soil horizons as an insoluble, but weatherable, biogenically precipitated crystalline form.

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