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Fungi, bacteria and soil pH: the oxalate–carbonate pathway as a model for metabolic interaction

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Summary

The oxalate–carbonate pathway involves the oxidation of calcium oxalate to low-magnesium calcite and represents a potential long-term terrestrial sink for atmospheric CO_2. In this pathway, bacterial oxalate degradation is associated with a strong local alkalinization and subsequent carbonate precipitation. In order to test whether this process occurs in soil, the role of bacteria, fungi and calcium oxalate amendments was studied using microcosms. In a model system with sterile soil amended with laboratory cultures of oxalotrophic bacteria and fungi, the addition of calcium oxalate induced a distinct pH shift and led to the final precipitation of calcite. However, the simultaneous presence of bacteria and fungi was essential to drive this pH shift. Growth of both oxalotrophic bacteria and fungi was confirmed by qPCR on the *frc* (oxalotrophic bacteria) and 16S rRNA genes, and the quantification of ergosterol (active fungal biomass) respectively. The experiment was replicated in microcosms with non-sterilized soil. In this case, the bacterial and fungal contribution to oxalate degradation was evaluated by treatments with specific biocides (cycloheximide and bronopol). Results showed that the autochthonous microflora oxidized calcium oxalate and induced a significant soil alkalinization. Moreover, data confirmed the results from the model soil showing that bacteria are essentially responsible for the pH shift, but require the presence of fungi for their oxalotrophic activity. The combined results highlight that the interaction between bacteria and fungi is essential to drive metabolic processes in complex environments such as soil.

Introduction

Oxalic acid ($H_2C_2O_4$) and oxalate minerals are major secondary products of plants, animals, fungi and bacteria present in soils (Tamer and Aragno, 1980). Calcium oxalate has been reported in more than 215 families of angiosperms and gymnosperms and occurs in the wood of more than 1000 genera of trees (Franceschi and Nakata, 2005), representing in some species more than 50% of their dry weight (Libert and Franceschi, 1987). Oxalate exudation in soils plays an important role by increasing the availability of phosphorous and micronutrients for plant uptake (Sahin, 2003).

Oxalotrophy, the metabolism of oxalate by bacteria, has been recognized as an important part of the biogeochemical carbon cycle as it allows the precipitation of calcium carbonate ($CaCO_3$) in acidic tropical soils, which are, otherwise, free of primary carbonates. This process is central to the oxalate–carbonate pathway (Fig. 1), which couples the biogeochemical cycles of calcium and carbon, and is gaining increasing interest as a potential long-term sink for atmospheric CO_2 (Braissant *et al*., 2002; Garvie, 2003; Cailleau *et al*., 2004; 2011).

Three factors are required for an operating oxalate–carbonate pathway: Ca^{2+}, oxalate and oxalate-degrading organisms. Oxalate degradation can be performed by a variety of plants and microorganisms (Dumas *et al*., 1995; Makela *et al*., 2002; Tuason and Arocena, 2009). However, in the case of its calcium salt ($K_{sp} = 2.32 \times 10^{-9}$), spontaneous oxidation of oxalate is highly unlikely because of the high activation energy required. Therefore, any metal oxalate can be considered as a compound in a metastable equilibrium (Verrecchia *et al*., 2006). Only bacteria are so far undoubtedly known to be able to participate in the oxidation. Oxalate catabolism by bacteria is also associated with a strong pH increase (Jayasuriya, 1955; Braissant *et al*., 2004), due to the conversion of a

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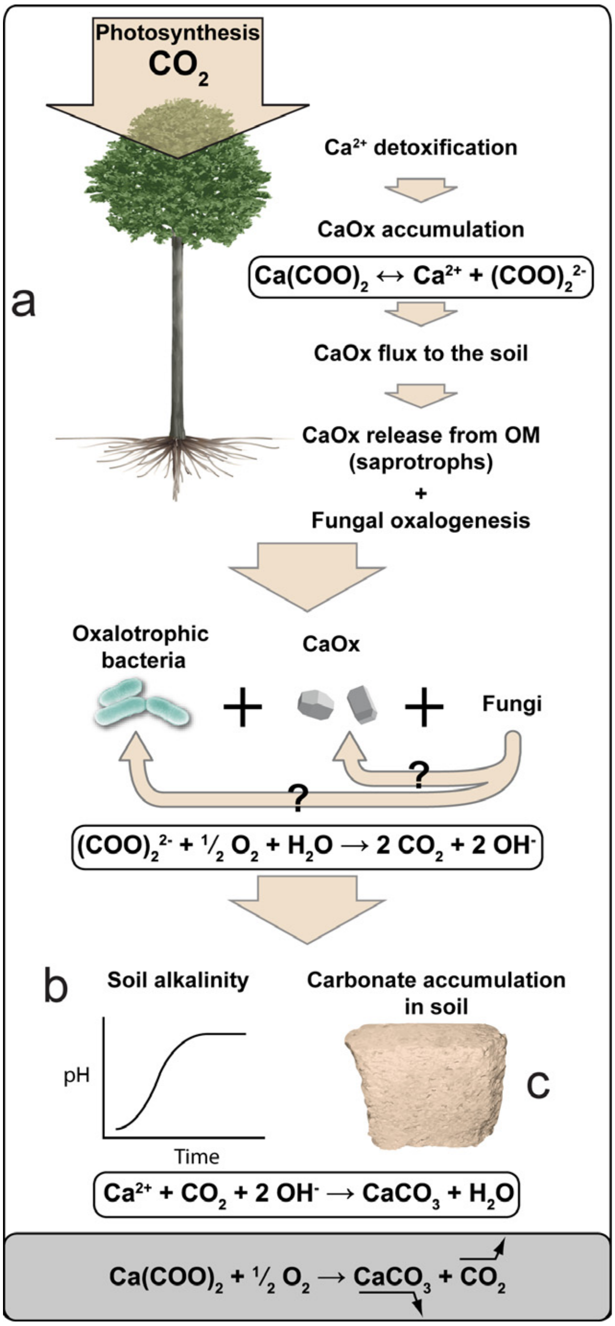

Fig. 1. Schematic representation of the oxalate–carbonate pathway showing the main biological players and the chemical reactions involved (modified from Aragno and Verrecchia, 2012). The unknown contribution of fungi as calcium oxalate producers and the role of their interaction with bacteria are indicated by a question mark. CaOx = calcium oxalate. a = processes leading to the formation of CaOx in the plant and fungi, oxidation of CaOx by bacteria and arrows indicating the unknown contribution of fungi; b = effect of oxidation of CaOx on soil pH over time; c = final product of the oxalate–carbonate pathway. The reactions leading to the formation of calcium carbonate are indicated below.

strong acid into a weaker one (Cromack *et al*., 1977). The influence of oxalotrophy on pH was first observed in faeces of plant litter detritivores (Van de Drift and Witkamp, 1960; McBrayer, 1973) and was deemed of interest for nutrient cycling, particularly of Ca and P (Cromack *et al*., 1977; Graustein *et al*., 1977).

Oxalotrophy is widespread and can be found in Gram-negative (*Alpha*-, *Beta*- and *Gammaproteobacteria*) and Gram-positive (*Firmicutes* and *Actinobacteria*) bacteria (Sahin, 2003). Thus, the study of the diversity and abundance of oxalotrophic bacteria cannot be based on a phylogenetic molecular marker such as the 16S rRNA gene. Instead, a gene directly involved in the metabolism of oxalate is a better candidate. At least two enzymes are involved in the catabolism of oxalate in aerobic and anaerobic bacteria. The first enzyme, the formyl coenzyme A (CoA) transferase, is encoded by the *frc* gene and transfers a coenzyme A moiety to activate oxalic acid (Sidhu *et al*., 1997). The second enzyme is an oxalyl CoA decarboxylase, encoded by *oxc*, which decarboxylates the activated oxalate molecule (Lung *et al*., 1994). Recently, specific primers targeting *frc* have been designed and tested in a variety of oxalotrophic bacteria and environmental samples, and can be used for diversity or quantification studies (Khammar *et al*., 2009).

Bacteria alone are sufficient to shift the pH from acidic to alkaline in cultures with calcium oxalate as sole carbon source (Jayasuriya, 1955; Braissant *et al*., 2002; 2004). Under these experimental conditions, the pH shift allows the precipitation of calcium carbonate crystals (Braissant *et al*., 2002). To date, however, there is no direct evidence showing that bacteria can oxidize calcium oxalate under natural environmental conditions and induce the pH shift required for calcium carbonate precipitation. Furthermore, although fungi are recognized as major players in the oxalate cycle in soils (Dutton and Evans, 1996; Tuason and Arocena, 2009), their role in the oxalate–carbonate pathway, and more generally in the functioning of soils, needs to be clarified.

Consequently, two major questions were addressed in this study: (i) can oxalotrophic bacteria alone cause the shift in pH required for the precipitation of calcium carbonate in soil? (ii) to what extent are fungi–bacteria interactions instrumental for the pH shift to occur? Microcosm experiments were conducted to fill the gap between experiments with pure cultures and field observations in order to answer these two questions. In a first experiment, a sterile soil was inoculated with a mix of pure bacterial and fungal cultures. In a second one, fresh soil collected near an oxalogenic tree and containing its own complex native microbial community was treated to selectively inhibit the activity of bacteria or/and fungi and to test their individual contribution to soil pH shift.

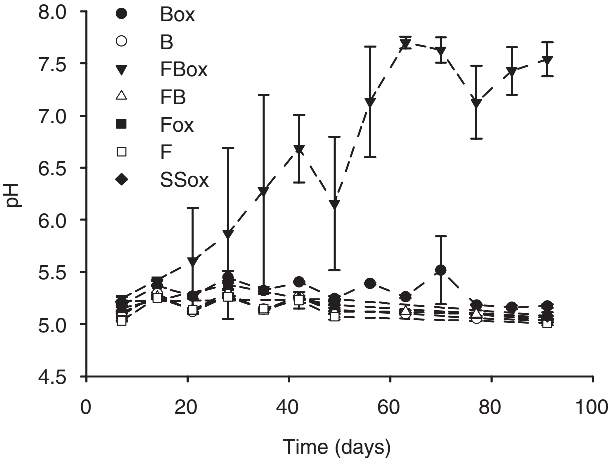

Fig. 2. Evolution of pH in microcosms created with a sterile soil and inoculated with allochthonous microbial community. The different treatments were: inoculation with bacteria and Ca-oxalate amendment (Box), inoculation with bacteria, without Ca-oxalate amendment (B), inoculation with bacteria and fungi, with Ca-oxalate amendment (FBox), inoculation with bacteria and fungi, without Ca-oxalate amendment (FB), inoculation with fungi and Ca-oxalate amendment (Fox), inoculation with fungi, without Ca-oxalate amendment (F), and finally sterile microcosms with Ca-oxalate amendment (SSox). Data points represent mean values of samples (± standard deviations) from three independent microcosms.

Results

Shift in soil pH by an artificial microbial community

A shift in soil pH towards alkaline conditions is a key element of the oxalate–carbonate pathway because it can trigger the precipitation of calcite in initially acidic soils (Braissant *et al.*, 2004; Cailleau *et al.*, 2004; 2005). We evaluated the influence of amendments with bacteria, fungi and oxalate on changes in soil pH in seven different microcosms (Fig. 2). For most of the systems assayed, pH values remained unchanged for more than 90 days. However, in the treatment amended with bacteria, fungi and oxalate (FBox), an increase in soil pH was observed after 20 days of incubation. After 90 days, soil pH had reached a final value of 7.5, being 2.5 pH units higher than the initial value. This coincided simultaneously with a decrease in the oxalate concentration from 34.2 ± 24.5 mg g^{-1} to 7.9 ± 5.2 mg g^{-1}. This pH shift was sufficient to induce calcite precipitation, which was not observed in treatments where the pH remained constant. X-ray diffraction analysis revealed small yet characteristic peaks for calcite in FBox soil but not in SSox soil (Fig. S1).

Development of inoculated bacteria and fungi in microcosms

Bacterial metabolism of oxalate has been shown to induce a pH increase in experiments on Petri dishes (Jayasuriya, 1955; Braissant *et al.*, 2004). Surprisingly, amendment of oxalotrophic bacteria was not sufficient to induce such a pH change in oxalate containing microcosms (Box). Addition of fungi alone (with or without oxalate) did not lead to a change in the soil pH either, raising the question as to whether the added microorganisms survived and developed in these microcosms. In order to test this, we followed copy numbers of *frc* and 16S rRNA genes of oxalotrophic and total bacteria, respectively, by qPCR (Fig. 3). As for soil pH, bacterial abundance increased only in the FBox treatment, where copy numbers of both marker genes increased by three to four orders of magnitude (*frc*: 3.7×10^4 to 2.8×10^8; 16S rRNA gene: 2.3×10^6 to 3.8×10^9). A statistical analysis shows a significant increase in *frc* copy number between 1 and 5, as well as 5 and 9 weeks (*P*-value = 0.0009 and 0.00006 respectively). In all other treatments containing bacteria (alone or in the presence of fungi), bacterial abundance remained close to the values recorded 7 days after inoculation (Fig. 3). In the bacteria-free microcosms,

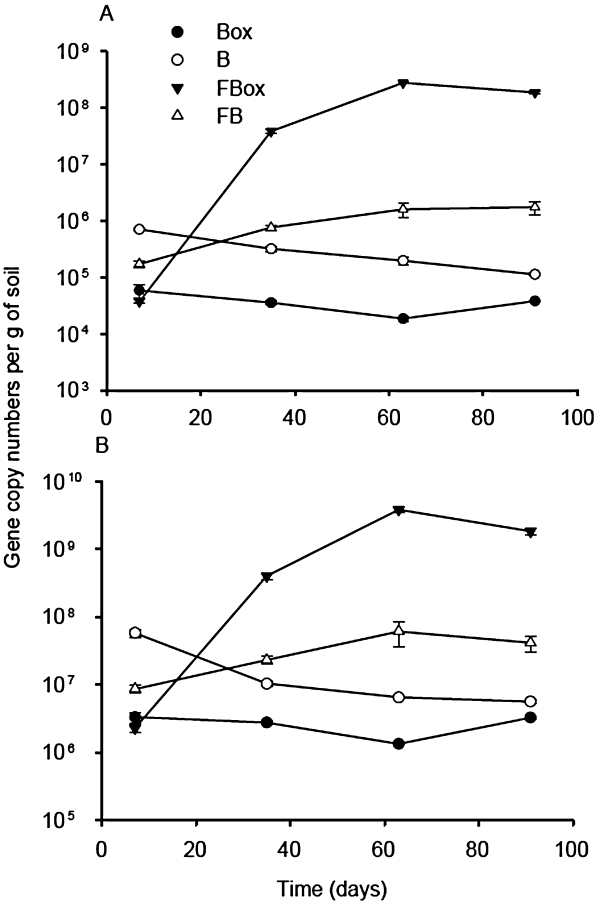

Fig. 3. Quantification of *frc* gene (A) and 16S rRNA gene (B) copy numbers by qPCR in microcosms with sterile soil and inoculated with allochthonous microbial community. Mean values and standard deviations of replicate quantification (*n* = 3) for three separate DNA extracts are given. For abbreviations see caption of Fig. 2.

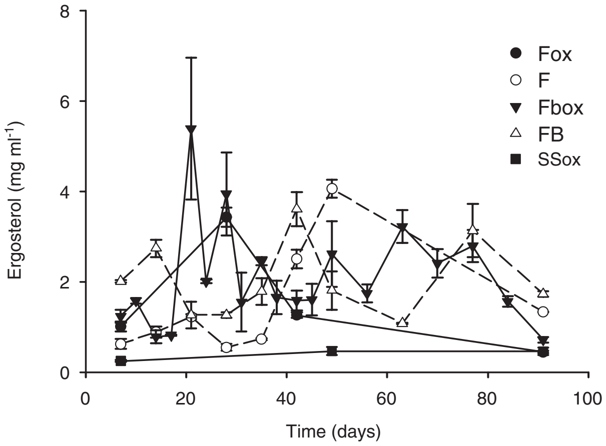

Fig. 4. Quantification of ergosterol in microcosms with sterile soil and inoculated with allochthonous microbial community. Mean values (± standard deviations) of the quantification for three separate analyses. For abbreviations see caption of Fig. 2.

frc gene concentration was below the detection limit (data not shown). Although the changes in the composition of the bacterial community were not the focus of this study, those could be indirectly observed based on the melting behaviour of *frc* PCR products (Fig. S2A). In absence of oxalate (B and FB), the melting temperature of the qPCR *frc* gene amplicons corresponded to those of the strains *Pandoraea* sp.*, Cupriavidus necator* and *Variovorax paradoxus* (approximately 87°C). In contrast, in systems amended with oxalate (FBox and Box), melting values changed considerably over time, suggesting changes in the community composition. In the Box treatment, the melting temperature of the amplicons did not correspond to any specific taxon. In the presence of fungi (FBox), the melting temperature of the amplicons decreased over time and was close to those of *Oxalicibacterium flavum* and *Streptomyces violaceoruber* (approximately 85°C). Changes in community composition in FBox were confirmed by DGGE (Fig. S2B). Results suggest that among the non-oxalotrophic strains, only *Escherichia coli* remained detectable for the first 35 days of incubation. For the oxalotrophic bacteria, *O. flavum* was constantly present until 63 days of incubation, whereas *S. violaceoruber*, *C. necator* and *Pandoraea* sp. were detected only at two of the three time points. In the final point (91 days), a not easily identifiable band was observed.

Active fungal biomass was assessed by the ergosterol content in soil. In contrast to bacteria, fungi developed in all microcosms independently of the presence of bacteria or oxalate (Fig. 4). However, in treatments containing fungi and bacteria (FBox and FB), ergosterol concentrations fluctuated over time compared with the treatments with fungi alone (Fox and F).

Shift in soil pH in microcosms with native microbial community

The results obtained for the microcosms with an artificially recreated microbial community prompted us to verify the findings in a less artificial system. Therefore, a second set of microcosms with tropical acidic soil containing a native microbial community was carried out. Previous tests, including cultivation of oxalotrophic bacteria and *frc* amplification, suggested that this soil harboured an active guild of oxalotrophic bacteria (data not shown). In these microcosms, the various treatments of the previous experiment were mimicked by the addition of domain-specific biocides and changes in soil pH were again used as proxy for an operating oxalate–carbonate pathway (Fig. 5). In microcosms with the native microbial community, the pH rose by about one unit within 2 weeks after calcium oxalate addition but remained unchanged in the treatments with the biocide mix (SSox analogue; Fig. 5). Furthermore, maximum pH (pH 8) was reached in less than 10 days as compared with 8 weeks in the first experiment (Fig. 2). A shift in soil pH was observed under two experimental conditions: no biocide treatment (FBox analogue) and cycloheximide-treated soil (Box analogue).

Effect of biocides on bacteria and fungi

The results from the microcosms with native microbial community (FBox analogue, Fig. 5) confirmed those of the first microcosm series (Fig. 2). However, the pH shift in the Box analogue suggests that the bacterial activity alone acted as the driver of soil pH shift for a native microbial community. In order to confirm the effect of biocides, the abundance of bacteria and fungi was determined at the end of the experiment. Although ergosterol was also measured, several additional peaks affected the interpretation of results and therefore fungal abundance

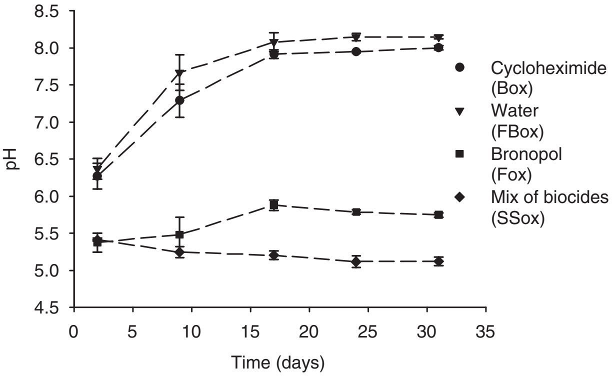

Fig. 5. Evolution of pH in soil microcosms with native microflora and treated with specific biocides. Values are means (± standard deviations) of measurements performed on three separate microcosms. For abbreviations in brackets see caption of Fig. 2.

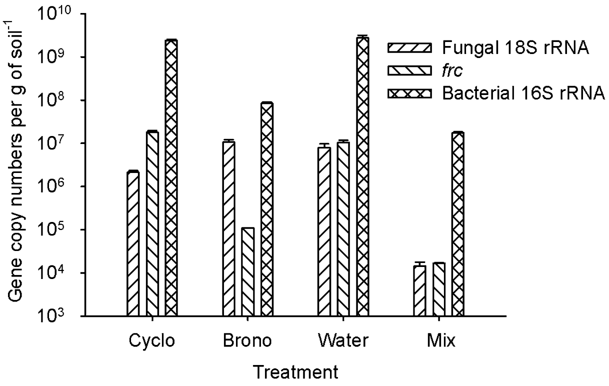

Fig. 6. Quantification of *frc*, 16S rRNA gene and 18S rRNA gene copies at the end of the experiment performed in soils with native microflora and treated with biocides. Values are means (± standard deviations) of quantifications performed with DNA from three replicate microcosms.

was estimated by qPCR amplification of the 18S rRNA gene.

The results for the FBox analogue system confirmed the presence of bacteria, oxalotrophic bacteria and fungi in this soil (Fig. 6). The bacterial and fungal abundances in this treatment were at least three orders of magnitude higher than in the control (SSox analogue). In the microcosms treated with bronopol alone (Fox analogue), bacterial abundance (total and oxalotrophic) dropped by one and a half orders of magnitude compared with the untreated soil (FBox). This system did not show a change in soil pH over time confirming the role of bacteria in the process. Finally, in the cycloheximide treated microcosm (Box analogue) fungal abundance decreased by less than an order of magnitude. A statistical analysis on the qPCR results shows that fungal abundance was significantly different between the water versus bronopol and cycloheximide treatments (P-value = 0.05), but not between water versus cycloheximide (P-value = 0.11), or bronopol (P-value = 0.54) alone. Therefore the cycloheximide treated microcosm (Box analogue) should be regarded as a less performing FBox analogue instead, still supporting the idea that bacteria and fungi are required simultaneously to cause the shift in soil pH observed in the oxalate–carbonate pathway.

Discussion

Experiments carried out with either an artificial or a native microbial community showed consistently that the simultaneous presence of bacteria, fungi and oxalate is essential to induce the alkalinization of the soil pH by up to 2.5 units and the precipitation of calcite, which are two keys effects observed for the oxalate–carbonate pathway in nature. These results confirm for the first time that the oxalate–carbonate pathway could be reproduced artificially in a microcosm, and that the parameters measured in the field (soil pH and presence of carbonates) effectively described an active pathway.

Although it is important to mention that the absolute *frc* copy numbers must be considered with caution due to methodological issues such as DNA extraction biases or the contribution of dead or lysed cells to the total DNA pool, the shift in soil pH was clearly associated with a significant increase of *frc* gene copy numbers of oxalotrophic bacteria, which supports earlier studies proposing that the metabolism of oxalate by bacteria may hold the key to pH increase (Cromack *et al*., 1977; Braissant *et al*., 2002; Cailleau *et al*., 2004). In pure cultures, bacteria alone cause oxalate oxidation and the associated pH increase (Sahin, 2003; Braissant *et al*., 2004). However, the situation is less straightforward in complex environments. In soils, bacteria needed the presence of fungi to thrive and oxidize calcium oxalate. In the case of the sterilized Ferralsol, it might be argued that the inoculated strains were not adapted to the acidic and nutrient poor conditions in the microcosm. However, in the presence of fungi (FBox), some of them were able to adapt and develop. In the Bolivian soil with its native bacterial community, adaptation is not an issue, which explains the faster kinetics of the pH shift, even though the native microbial communities could have changed during transport and storage of soil prior to the experiment. A fungal-free microcosm (Box analogue) was intended by applying cycloheximide. As suggested previously, this fungicide had probably only a limited effect, (Griffin *et al*., 1978; Sugiura *et al*., 1999), which may explain the lack of a strong difference between the treatments with cycloheximide (Box analogue) and water (FBox analogue). Considering that we cannot rule out some fungal activity in the microcosm treated with cycloheximide, the most conservative interpretation of the results suggest that the simultaneous presence of bacteria and fungi was required in all the microcosms showing a soil pH shift. In the case of bronopol, it has been reported as a biocide with a broad spectrum of antibacterial activity (Shepherd *et al*., 1988). From the results obtained, it inhibited 97% of the bacterial community in the soil assayed, and it has as a synergistic effect in combination with cycloheximide (99.4% fungal and bacterial inhibition). The reasons for this synergistic effect are unknown. Although non-target effects of bronopol are known, it appears not to affect fungal growth and thus the results are surprising (Rousk *et al*., 2008).

Recent studies suggest that interactions between bacteria and fungi contribute to the shaping of biological communities above and below Earth's surface (Boer *et al*., 2005; Nazir *et al*., 2010). However, we still know little about the nature of this interaction and its effect on nutrient, niche or habitat exploitation. The mechanisms by which fungi interact with bacteria vary from one situation to another.

For example, fungal hyphae can act as transport vectors in unsaturated porous media such as soils. Bioremediation studies have shown that in a heterogeneous and complex environment, fungi act as highways for active bacterial dispersal towards pollutants (Kohlmeier *et al*., 2005; Wick *et al*., 2007; Furuno *et al*., 2010). Moreover, ecological studies have revealed specific interactions between fungal and bacterial species (Warmink *et al*., 2009; Warmink and van Elsas, 2009), as well as a 'helper' effect of some migratory bacteria on non-migratory bacteria (Warmink *et al*., 2011). We postulate that in our experiments fungi permitted a better and faster colonization of the microcosms by serving as a fungal highway and providing better access to the highly insoluble substrate Ca-oxalate. This is a key issue since the structure of soils used for the experiments were undoubtedly altered prior to inoculation or addition of calcium oxalate (e.g. drying, sieving and re-watering), making probably harder the dispersion of bacteria. We tested the bacterial and the fungal strains used in the amendment experiments for such a dispersion mechanism and found that *Pandoraea* sp., *Ancylobacter polymorphus* and *C. necator* were able to migrate from an unfavourable (straw) to a favourable substrate (nutrient agar) by crossing the liquid-air barrier with the aid of fungal hyphae (Fig. S3).

Facilitating access to nutrients, provision of growth factors and organic compounds (i.e. fungal exudates), as well as modification of the microenvironmental conditions, represent further beneficial effects of fungi on bacterial development. It has been suggested that soil fungi can affect the activity of fungi-associated bacteria by secretion of stimulatory or inhibitory compounds, or by changing soil structure (Johansson *et al*., 2004). Fungi have been shown to protect bacteria against acidic pH (Warmink and van Elsas, 2009), which could have been a critical factor in the amendment experiments with acidic Ferralsol, since soil bacteria need to be adapted to the natural pH of their habitat (Baath, 1996; Fernandez-Calvino and Baath, 2010). Consequently, we tested the effect of acidic pH on the growth of the chosen oxalotrophic strains. With the exception of *Pandoraea* sp., none of them grew on solid media at pH 5. At least, two additional species (*A. polymorphus* and *C. necator*) could grow on the same medium when grown in co-culture with *Pycnoporus cinnabarinus*, suggesting that this protective role of fungi may have played a role in the microcosms as well (Table S1).

In addition to the positive effects mentioned above, recent studies have suggested that fungi compete successfully with bacteria for the colonization of specific habitats. For example, white-rot fungi out-competed bacteria for the colonization of sterile beech wood blocks (Folman *et al*., 2008). In that study, a selective stimulation of specific bacterial taxa, and particularly of potentially oxalotrophic bacteria, was observed on the fungal cords. This specific stimulation can be due to the fact that some white-rot fungi precipitate calcium oxalate crystals on the hyphal surface (Tuason and Arocena, 2009). Indeed, we have tested experimentally the production of calcium oxalate crystals by the three fungal species used in this study. All the species were able to produce calcium oxalate crystals, potentially providing an additional substrate for bacteria (Guggiari *et al*., 2011). However, the production of calcium oxalate of fungal origin during the experiments was not tested.

The oxalate–carbonate pathway is considered as a promising natural process for trapping atmospheric carbon. This study provides the first key towards optimizing the system in a natural environment. It confirms the role of bacteria as major calcium oxalate oxidizers. It also demonstrates that soil bacteria are able to adapt and to respond rapidly to a significant input of calcium oxalate despite the potential stress induced by it. Furthermore, fungi–bacteria interactions also appear to be a key factor for the development of *frc*-positive bacteria under stress conditions. The understanding of this interaction, however, is still in its infancy and should be further explored in order to enhance the efficiency and the potential value of the oxalate–carbonate pathway worldwide. The observation of complex fungi–bacteria interactions in the oxalate–carbonate pathway may represent general principles in microbial ecology and thus be of great interest for other fields of basic and applied research such as pest control, crop yield improvement by inoculation of plant growth promoting bacteria or bioremediation.

Experimental procedures

Bacterial strains and growth conditions

All bacterial strains are from the culture collection of the Laboratory of Microbiology at University of Neuchâtel. Nine bacterial species were selected. Six strains (*Pandoraea* sp., *O. flavum* T, *A. polymorphus*, *S. violaceoruber*, *C. necator* and *Alcaligenes paradoxus* T) grew with oxalate as sole carbon source (Tamer and Aragno, 1980) and were positive for the amplification of the *frc* gene (Khammar *et al*., 2009). These species were considered to be oxalotrophic. Two species (*Bacillus subtilis, Pseudomonas aeruginosa*) were unable to oxidize oxalate and lacked the gene *frc*. *Escherichia coli* K12 was unable to oxidize oxalate (non-oxalotrophic), but was positive for the presence of the *frc* gene (Table 1). Bacteria were cultivated in 1 l Schott^{TM} bottles containing 400 ml of the appropriate medium (Table 1) until late exponential phase. For the preparation of the inoculum, cells were harvested by centrifugation at 5000 *g* and 4°C for 15 min (*O. flavum* T, *Pandoraea* sp. and *S. violaceoruber* were centrifuged for 1 h), and then washed with sterile physiological salt solution (NaCl 0.9%). For each strain, cell suspensions with 8.3×10^8 cells per ml were prepared separately, then mixed in equal

Table 1. Bacterial and fungal strains used for the inoculation of microcosms**.**

Ref. No.	Strain	Oxalotrophy	*frc* gene	Culture medium	Melting temperature of *frc* amplicon (°C)
23778	*Bacillus subtilis*	–	–	NB	NA
45*	*Pandoraea* sp.	+	+	NB	86.9
15506	*Oxalicibacterium flavum* T	+	+	ST1 + 1% glycerol	85
1007*	*Escherichia coli*	–	+	NB	84.1
1023*	*Pseudomonas aeruginosa*	–	–	NB	NA
18745	*Ancylobacter polymorphus*	+	+	NB	85.9
40783	*Streptomyces violaceoruber*	+	+	NB + 0.1% SDS	85.1
428	*Cupriavidus necator*	+	+	NB	86.9
30034	*Variovorax paradoxus*	+	+	NB	87.1
NA	*Pycnoporus cinnabarinus*	NA	NA	*Miscanthus* straw	NA
NA	*Trametes versicolor*	NA	NA	*Miscanthus* straw	NA
NA	*Polyporus ciliatus*	NA	NA	*Miscanthus* straw	NA

Ref. No. is for the reference numbers of the strain in the German Collection of Microorganisms and Cell Cultures (DSMZ), or in the in the culture collection at the University of Neuchâtel (*). NB, nutrient broth; ST1, standard medium 1 (Merck, Darmstadt, Germany); SDS, sodium dodecyl sulfate. Last column gives the value of the melting temperature of *frc* gene qPCR amplicons. NA, not applicable.

amounts, and finally added to the microcosms to a final concentration of 10^7 cells per species and per gram of soil.

Fungal strains and growth conditions

Three species of fungi, *P. cinnabarinus*, *Trametes versicolor* and *Polyporus ciliatus* were used as inoculum. They were maintained on malt agar plates. They were grown on *Miscanthus* sp. straw for 3 weeks at 25°C for inoculation of the microcosms. Then, 2 cm^3 of colonized straw were cut, placed on top of the soil and covered with sterile *Miscanthus* sp. straw for fungal nutrition. Sterile *Miscanthus* sp. straw was also included in microcosms without fungi. Each of the three strains was placed at an equal distance from each other.

Origin and characterization of soil samples

The first soil corresponded to an A0 horizon of a Ferralsol (Lismore, NSW, Australia), which was selected because of its acidic pH, absence of carbonates, and lack of an actively occurring oxalate–carbonate pathway. It was used as a model soil to conduct 'additive' experiments with an allochthonous microbial community. Before the experiment, the soil was sieved at 2 mm, autoclaved twice (25 min at 121°C), and gamma irradiated at 48.4–54.3 kGy (Studer AG Werk Hard, Däniken, Switzerland). Soil sterility was confirmed by inoculating a few soil aggregates in 20 ml of nutrient broth (Biolife, Milano, Italy). After sterilization, final soil pH was 5.2 and its water holding capacity 0.45 ml g^{-1}. According to its texture, the soil from Australia was classified as a silty-clay soil, consisting of 45% clay and 51.6% silt. The mineralogical composition of the soil consisted of quartz, gibbsite, kaolinite and pumpellyite.

The second soil was a Cambisol collected under the canopy of the oxalogenic tree *Ceiba speciosa* (Malvaceae) in the region of Sapecho (Bolivia) at 10 cm in depth and 20 cm from the tree trunk. This soil corresponded to the B horizon of a Cambisol (Dystric). The soil was transported for 2 weeks before arrival to the laboratory, time during which it was stored at room temperature. Upon arrival in the laboratory, the soil was sieved (2 mm mesh) and stored for four additional days at 4°C in order to preserve its natural microbial community. The relative humidity corresponded to 1.3% (98.7% dry material). Soil pH at the beginning of the experiment was 5.5 (pH_{KCl} 4.4) and the water holding capacity 0.29 ml g^{-1}. The concentration of oxalate initially was 0.40 mg kg^{-1}. The soil texture consisted of 64.5% silt, 21.8% sand and 13.8% clay. The mineralogical composition of the soil consisted of quartz, phyllosilicates, mica, feldspath, kaolinite, plagioclase, goethite, haematite and some traces of magnesium calcite.

Microscosm design

Microcosm experiments were conducted in autoclavable 375 ml plastic containers (Magenta V 8505; Sigma-Aldrich, Germany). The lids were perforated twice. A central hole of 0.5 cm in diameter allowed the insertion of a 0.2 μm filter (X 50 Analypore PES 33 mm 0.2 μm, Fischer Scientific, Switzerland) for regular watering. A second hole with a diameter of 1.5 cm was covered with a cellulose stopper (16 mm top diameter, Semadeni, Switzerland) for aeration. Filters and stoppers were tightly sealed with silicone to prevent any contamination. All microcosms were filled with 50 g of soil.

Experimental design and sampling

Seven different combinations (in triplicates) were set up for the experiments. The Australian soil was inoculated with bacterial and fungal cultures as follows: bacteria and oxalate (Box), fungi and oxalate (Fox), bacteria, fungi and oxalate (FBox), bacteria only (B), fungi only (F) and fungi and bacteria (FB), and finally, only with oxalate (i.e. organism-free, SSox). Oxalate (4 mg g^{-1}) was added as calcium oxalate monohydrate (Acros Organics, Geel, Belgium) and mixed with the soil before addition of bacteria and fungi. This amount of oxalate used, which equal to the concentration used for the enrichment and isolation of oxalotrophic bacteria from soils (Tamer and Aragno, 1980), is higher than the

Table 2. Primer sets and amplification conditions for the qPCR analysis of the *frc*, 16S rRNA and 18S rRNA genes**.**

Gene	Primers	Primer concentration (μM)	DNA concentration (ng μl^{-1})	Program	Reference
frc	F: 5′-CTSTAYTTCACSATGCTSAAC-3′ R: 5′-GDSAAGCCCATVCGRTC-3′	1.25	1.6–4	40 cycles of 30 s–95°C; 60 s–56°C; 30 s–72°C	Khammar *et al.* (2009)
16S rRNA gene	F: 5′-ACTCCTACGGGAGGCAGCAG-3′ R: 5′-ATTACCGCGGCTGCTGG-3′	0.3	1.6–4	35 cycles of 10 s–95°C; 15 s–55°C; 20 s–72°C	Muyzer *et al.* (1993)
18S rRNA gene	F: 5′-GTA GGT GAA CCT GCR G-3′ R: 5′-CGC TGC GTT CTT CAT CG-3′	0.3	1.6–4	35 cycles of 10 s–95°C; 15 s–55°C; 20 s–72°C	Lopez-Garcia *et al.* (2001)[a] Fierer *et al.* (2005)

a. Modified by using primer in reversed complement orientation.

values found in nature that range from 0.015 to 0.175 mg g^{-1} in soil (Braissant *et al*., 2002; Cailleau *et al*., 2004) to 1.3 mg g^{-1} in litter (G. Cailleau, unpublished). However, a higher oxalate concentration was selected because it allowed reproducing and stimulating a phenomenon that in nature takes several tens of years. Incubation was carried out at 25°C in the dark. The water content was adjusted weekly to 30% of the soil's holding capacity.

Three microcosms of each treatment were sampled in a destructive manner under sterile conditions at each sampling point. Soil was homogenized mechanically using a sterile weighing spoon. For DNA analysis, 4.5 g of soil were sampled and kept at −80°C until DNA extraction. Three samples of 15 ml were kept at −20°C for physicochemical analyses. After 7 weeks of incubation, all remaining microcosms were opened for 10 min under sterile conditions to ensure gas equilibrium with the atmosphere and to avoid accumulation of CO_2.

The experiment with native microbial communities (Bolivian soil) was designed to be as similar as possible to the first one, yet using a subtractive approach instead of an additive one. The bacteria only, fungi only and sterile treatment were mimicked by using 1 mg of a specific biocide per gram of soil with cycloheximide (Box analogue; Sigma-Aldrich, St. Louis, MO, USA; Ingham and Coleman, 1984), bronopol (2-Bromo-2-nitro-1,3-propanediol, Fox analogue; Sigma-Aldrich; Bailey *et al*., 2003; Rousk *et al*., 2008), or a combination of both (SSox analogue) respectively. Finally, a series of microcosms was prepared without biocides (FBox analogue). Biocides were dissolved in sterile deionized water and applied to the soil, which was incubated for 24 h at room temperature to allow biocides to act. After the incubation, 2 g of calcium oxalate monohydrate were suspended in the watering solution before its addition to facilitate dispersion. At this point, sterile water was added to reach 30% of the soil's water holding capacity and the first sampling (0 days) was carried out. Incubation was carried out at 25°C in the dark. The water content was adjusted weekly to 30% of the soil's holding capacity. Watering included the repeated addition of the appropriate biocide. For each treatment (triplicates) sampling was carried out in a non-destructive way every week after watering. Approximately, 2 g of soil were sampled for each microcosm and kept at −80°C. Soil pH was monitored using a HELLIGE® Soil Reaction pH Tester (Ben Meadows, USA) and the experiment was terminated once the soil pH stabilized.

Soil pH measurements

Soil was dried overnight at 105°C. A volume of 1.25 ml of deionized water was added to 0.525 g (± 0.025 g) of soil, placed on a rotary shaker for 2 h and centrifuged at 16 000 *g* for 1 min. Soil pH_{H2O} was selected for the experiments because it is supposedly of higher relevance for biological systems (Gobat *et al*., 2004). However, pH_{KCl} values were also determined and corresponded to 0.2–0.3 pH units above the pH_{H2O} values measured (data not shown). The pH was measured in the supernatant with a pH microprobe (Biotrode, Metrohm, Zofingen, Switzerland). The significance of the difference between values was assessed using bilateral Student's *t*-test.

DNA extraction

DNA was extracted using the FastDNA® Spin Kit for Soil (Qbiogene, Irvine, CA, USA) with a Fast-Prep™ bead-beating device (FP 120, Savant Instruments, HotBrook, NY, USA). Extractions were done according to the manufacturer's instructions. DNA extracts were quantified using a Nanodrop® spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, USA) and conserved at −20°C. DNA concentration ranged from 6 ng μl^{-1} to 165 ng μl^{-1}. Samples were diluted to 1/50 to limit the inhibitor effect of co-extracted soil humic acids. This 1/50 dilution factor was chosen experimentally after testing different dilutions (1/10 to 1/200) of soil DNA extract for the inhibition of qPCR on a constant quantity of a DNA solution of plasmid including a gene (*ascV*) absent in the Australian microcosms. The 1/50 dilution led to the highest Ct and an accurate quantification and therefore was chosen for all the soils. For the first set of microcosms, three DNA extractions were performed and analysed as individual replicates. For the second set, DNA was extracted from three individual microcosm replicates.

Quantification by qPCR

Quantification of the *frc*, 16S rRNA and 18S rRNA genes was carried out using the QuantiTect SYBR® Green PCR Kit (Qiagen®, Hilden, Germany). The experimental conditions varied slightly for each gene target (Table 2). PCR was run on a Rotor-Gene™ 6000 instrument. The fluorescence data were analysed with the Rotor-Gene 6 software (Corbett research, Sydney, Australia). Thresholds (Th), Ct values and derivatives of melting curves were determined using Rotor-Gene 6 software. For each gene, all extracts were analysed in a single qPCR run in order to minimize experimental error (Smith and Osborn, 2009). For quantification, three independent standards series with 10^3 to 10^7 gene copies μl^{-1} were included. Standards were prepared with known amounts of plasmid DNA containing a fragment of the target gene. Obtained gene copy numbers in samples were corrected for differences in DNA extraction efficiencies by normalizing values to per gram of soil units. The significance of the difference between values was assessed as mentioned for the pH measurements.

Quantification of ergosterol

The method was adapted from Young (1995) and Larsen and colleagues (2004). Briefly, 4 ml of methanol and 1 ml of 2 N NaOH were added to 2 g of lyophilized soil and mixed by vortexing. The solution was placed in a water bath at 85°C for 30 min. After rapid cooling, 1 ml of 2 N HCl was added. The tubes were centrifuged at 400 r.p.m. for 2 min and the supernatant transferred to new tubes. Two millilitres of pentane was added for ergosterol extraction. Tubes were shaken manually and the liquid transferred to HPLC vials (Infochroma AG, Switzerland). The contents of the flasks were evaporated under N_2 flow using a Techne concentrator Dri-Blocks dB 3D (Techne, USA) at 37°C. The vials were preserved in the dark at –20°C. Before analysis, 100 μl of methanol was added. Chromatography (20 μl injection volume) was performed at 35°C on an EC 250/4 Nucleosil 100-5 C18 column (Macherey-Nagel, Oensingen, Switzerland) using a 95:5 v/v mixture of methanol and acetonitrile as mobile phase. Using a pump rate of 1 ml min^{-1}, ergosterol eluted after 8 min and was detected at 280 nm. Ergosterol (Sigma) stock solution and standards were prepared in methanol.

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Supporting information

Additional Supporting Information may be found in the online version of this article:

Fig. S1. XRD analysis showing the precipitation of calcite in the FBox treatment (down panel) compared with the sterile soil in presence of oxalate (SSox treatment; upper panel). Mineralogical composition determinations were performed using a Scintag diffractometer. X-ray diffractograms were analysed using Macdiff software V5.4.1. In the lower panel (FBox) only the signature for calcite is indicated. The other major components (quartz, gibbsite, kaolinite and pumpellyite) are also present but are not indicated.

Fig. S2. Changes in the composition of bacteria in the microcosms experiment with the Australian soil.

A. Melting curves of *frc* qPCR products obtained at different sampling time points. The melting analysis was carried out at the end of the *frc* qPCR by increasing the temperature from 72°C to 95°C.

B. DGGE analysis of the 16S rRNA gene for the FBox treatment at different time points. For DGGE the nearly complete 16S rRNA gene was amplified using the general bacterial primers GM3f and GM4r (Muyzer *et al*., 1995). The products were cleaned using a multiscreen plate (Millipore) and diluted 100 times to be used as template for a nested PCR amplification with the primers P3 (GC-clamped) and P2 (Muyzer *et al*., 1993). A touchdown temperature programme was used for nested PCR. A DCode System (Bio-Rad) was used for DGGE of the 16S rRNA gene PCR products. Separation was carried out in 7.5% polyacrylamide gels with a gradient of 35–65% of denaturants (100% denaturants contained 420 g l^{-1} urea and 400 ml l^{-1} deionized formamide in 0.5× TAE) during 5 h at 150 V and 60°C. Gels were stained with GelRed (BioTium). The ladder (L) consisted of 16S rRNA sequences from *Pandoraea* sp. NEU 45 (1), *Oxalicibacterium flavum* DSM 15507 (2), *Ancylobacter polymorphus* DSM 18745 (3), *Streptomyces violaceoruber* DSM 40783 (4), *Methylobacterium thiocyanatum* NEU 1216 (5), *Cupriavidus necator* DSM 428 (6), and *Escherichia coli* NEU 1007 (7).

Fig. S3. Dispersion of the bacterial strains used for the microcosm experiment with Australian soil using the fungal highway.

A. Schematic representation of the experimental set-up. An inverted Petri dish containing nutrient agar (NA) as target medium and straw (on the cover) is used for the inoculation of bacteria alone or a combination of bacteria and fungi. To access the target medium bacteria must cross the air space between the inoculation and target medium. In the case of a fungal highway, hyphae provide a surface that can be used by bacteria to carry out the crossing.

B. Formation of fungal cords crossing the medium-air barrier between the straw and the target medium NA.

C. Growth tests carried out using bacteria alone (right panel) or in combination with the three fungal strains (left panel) listed in Table 1. 1. *Bacillus subtilis*; 2. *Pandoraea* sp.; 3. *Oxalicibacterium flavum*; 4. *Escherichia coli*; 5. *Pseudomonas aeruginosa*; 6. *Ancylobacter polymorphus*; 7. *Streptomyces violaceoruber*; 8. *Cupriavidus necator*; 9. *Alcaligenes paradoxus*.

Table S1. Effect of pH on growth of the oxalotrophic bacterial strains used in the microcosm experiments in both presence and absence of *Pycnoporus cinnabarinus*. The specific medium used previously was prepared with 1.3% agar and HCl was added to set the pH. Growth was tested simultaneously at pH 7 and 5. Strains unable to grow at pH 5 were then tested for their ability to overpass the pH stress if grown in the presence of *P. cinnabarinus*. For that purpose, the same media as described above were prepared. Prior to inoculation a circular cavity was prepared in the middle of each Petri dish, which was filled with sterile M*iscanthus* sp. straw. The fungus was cultured previously in malt agar (12 g l^{-1}, Biolife, Milan, Italy) and transferred to the straw. Then, the bacterial strains were inoculated and incubated at 30°C in the dark. Growth was checked every 24 h for 20 days. For those strains that grew in the presence of the fungus the number of days required for observing colony formation is indicated in brackets. N.D., not determined.