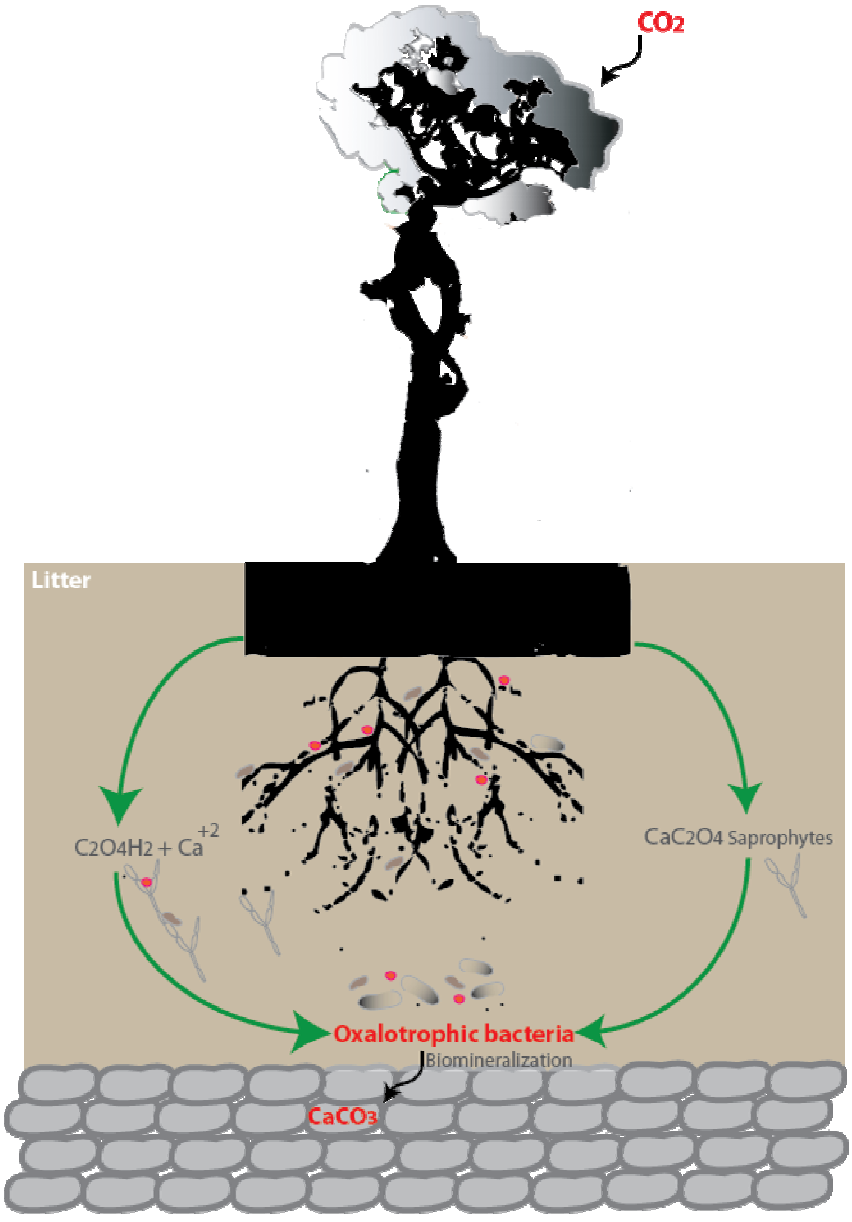
Daniel Bravo

Assessing the diversity and metabolism of oxalotrophic bacteria in tropical soils



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Assessing the diversity and metabolism of oxalotrophic bacteria in tropical soils

Faculty of Sciences

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Summary

Climate change is increasing as a consequence of elevated concentrations of CO_2 in the atmosphere. Among the scientific strategies proposed to tackle the effect of elevated CO_2, the biogeochemical oxalate-carbonate pathway (OCP) occurring in terrestrial habitats, appears to be important since it is considered as a potential carbon sink. This process occurs naturally in Earth, with a particular importance in tropical forests. Previous studies were carried out in a subtropical forest to evaluate the precipitation and biomineralization of carbonate due to biologic (microbial oxalotrophy) activity. However, the knowledge about biodiversity and the metabolic rates of oxalate consumption, as well as the effect of microbial interactions over the OCP at several tropical soils was poorly described. Therefore, the aim of this thesis was to assess the diversity and metabolism of oxalotrophic bacteria related with the OCP in tropical habitats. This document represents the first detailed study about metabolism and diversity of oxalotrophic bacteria found in three tropical soils in Bolivia, Indian, and Cameroon, implicated in oxalate-carbonate transformations. At those sampling sites, oxalate producing trees (oxalogenic trees) were assessed by biotic and abiotic treats influencing the pathway occurring there. The manuscript is organized in seven chapters. The first two chapters deal with the development and application of analytical and molecular techniques, such as isothermal microcalorimetry (IMC) and BrdU labeling DNA - DGGE, to study metabolism and diversity of model and active environmental oxalotrophic bacteria. The relevance of active oxalotrophic bacteria is highlighted. For instance, the ecological role of relatives to *Kribbella* phylotypes and related actinobacteria within the oxalotrophic group found in Cameroon is discussed at the end of chapter three. Moreover, the following chapter deal with a collection of oxalotrophic bacteria isolated from soil samples recovered in field trips performed at Bolivia, India, and Cameroon, where oxalogenic trees were found. The chapter includes a complete characterization of ten oxalotrophs, with the interest to understand their capability to consume oxalate as sole carbon and energy source, as well as, their metabolic plasticity by the consumption of other carbon substrates. High oxalate consumption rates were observed for strains such as *Variovorax soil* C18, *Lysobacter* sp. A8, *Agrobacterium* sp. B23, and *Streptomyces achromogenes* A9. Chapter five describe the development of a new technique of isolation of autochthonous couples of bacteria and fungi from soil implied in oxalotrophy. The case of the fungus *Trichoderma* sp. and eight oxalotrophic bacteria obtained from soils samples influenced by oxalogenic trees in Cameroon is discussed. Furthermore, a global discussion including a comparison of abundance and vertical distribution of oxalotrophic bacteria through soil profiles influenced by oxalate-producer trees is part of the chapter six. Biotic and abiotic treats are correlated statistically to compare and understand the OCP systems occurring in India and Cameroon. The contribution to the knowledge in oxalotrophic diversity and metabolism relative to the oxalate-carbonate pathway occurring in tropical soils, as well as, the perspectives of this work in CO_2 management are presented at the end of chapter six. In chapter seven is exposed a complete revision of the interactions between soil pH, fungi and bacteria as key actors at the geo-biological interface of the pathway, put it all in evidences through microcosms experiments.

Dedicado a mi abuela („Abue") y a Mamá (Clarita).

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Chapter 1. General Introduction

Chapter summary

This section introduces the context of the current doctoral thesis. The aim of this introduction is to give the reader the tools to understand the neural concepts of this study, such as climate change -a hot issue in environmental sciences-, microbiology -an important area in life sciences-, and microbial ecology -trying to go beyond the description of biological players in nature-. Finally the aims of this thesis are described.

Chapter 1. General Introduction to the Oxalate-carbonate pathway (OCP) and the diversity of oxalotrophic bacteria

a. Climate change and global warming

How important is to study climatic change and global warming?

Climate change and global warming should radically changed our life style and priorities at each level, which includes the focus of current scientific research. The complex environmental problems associated to drastic climatic changes imply to consider many strategies to resolve such issue, because several factors are involved, from individual social behavior, science, and engineering, to politics and economy. At the scientific level, the problematic of climatic change has increased considerably interdisciplinary work to figure out possible solutions to stop the increasing limiting conditions of Earth. Therefore, the present study is framed in the context of one of the less understood strategies, located at the interface between geological and microbiological approaches to tackle the effect of climatic change in terrestrial ecosystems such as the tropical forest. The present investigation aims at improving our understanding of certain phenomena at the microbiological level implied in the functioning of a particular biogeochemical cycle such as the oxalate-carbonate pathway (OCP), which would be useful to design further ideas as part of a possible geo-biological solution to tackle climate change.

However, to understand how a microbiological approach can be of interest to provide a possible solution to climate change in the context of terrestrial ecosystems, it is necessary to explain the origin and the implications of such a problem. Is also important to explain how to regulate atmospheric carbon dioxide (CO_2) levels, and how the OCP could serve to such purpose. Thus, in that way, study the role of oxalotrophic bacteria in that particular biogeochemical process becomes fundamental.

Due to human activities and the industrial revolution, the atmospheric concentrations of CO_2 have increased from 280 to 379 ppm in the past 150 years (Archer*, et al.*, 1995). The increase of pCO_2 has negative consequences for life on Earth, for example, decreasing the life quality, the basic renovable resources, and the biodiversity. The most noted effect of human activity is the phenomenon of global warming linked to a greenhouse effect (Robertson & Grace, 2004).

Nowadays, the increase in atmospheric CO_2 concentrations rose from 379 ppm in the eighty's of the last century to about 390 ppm in 2010 (Domenech*, et al.*, 2011) -year in which I started my PhD-. The resulting increase in temperature due to radiative forcing, a measurement of the quantity of solar radiation that arrive to the stratosphere and therefore are not deflected by the ozone layer, was in the range of 0.10 – 0.16°C. As a consequence, an increase over 1.6°C has been detected in the last seven years at a global scale, whereas an increase of 2.1°C has been detected at local scenarios in tropical habitats. Why such a difference? Tropical forests are located in the geographical zones in which solar exposition is greater than that detected in temperate zones. For this reason several strategies have been suggested to tackle the effect of global warming as consequence of the accumulation of CO_2 in the tropical zones (Raupach*, et al.*, 2007). Nevertheless, the effect of climatic change is extended to the whole planet, so that, assessing possible solutions at local scenarios like tropical soils, would involve tackling the problem at the global scale. Therefore, one of the potential useful strategies to reduce the concentrations of atmospheric CO_2 is the use of the biogeochemical cycle such as the oxalate carbonate pathway (OCP). The next section introduces the OCP and shows the importance of OCP systems in tropical forests.

b. The oxalate-carbonate pathway (OCP) in terrestrial environments

How important is the OCP in terrestrial environments in the context of climatic change and global warming?

In the context of climatic change, the oxalate-carbonate pathway is very important in terrestrial ecosystems in several ways. The OCP is a natural pathway involved in the terrestrial carbon cycle (Figure 1), which can contribute to the auto regulation of atmospheric CO_2 by exploiting the interaction of plants and microorganisms (Cailleau*, et al.*, 2011).

Especially, bio-mineralization processes carried out by a group of soil bacteria that can grow using oxalate as sole carbon and/or energy sources allowing $CaCO_3$ precipitation (Verrecchia*, et al.*, 2006). This pathway remains relatively unknown. The relationships with the current environmental problems discussed in the previous section. Before the euphoric search of strategies to tackle climate change, there were no theoretical models to explain the OCP, its consequence, and the transformations occurring in soil (Verrecchia*, et al.*, 2006).

The OCP primarily leads the accumulation of calcium carbonate ($CaCO_3$) or others carbonate forms (Cailleau*, et al.*, 2004) mainly depending of the oxalate salt oxidized. The formation of

calcite or calcium carbonate in acidic carbonate-free soils upon the biological degradation of oxalate and a concomitant alkalinization of soil has been pointed out as an underestimated long-term carbon sequestration mechanism (Braissant*, et al.*, 2004). In other words, the oxalate-carbonate pathway allows the conversion of atmospheric CO_2 into calcite stones in an efficient way (stable geomorphs) and at long term, at geological time (Braissant*, et al.*, 2004). This pathway also contributes to the fertilization of the same soils by the activation of other (or the same) functional bacterial groups, for example those responsible for the solubility of inorganic phosphates, or those responsible to Al detoxification of particular niches (Klug & Horst, 2010).

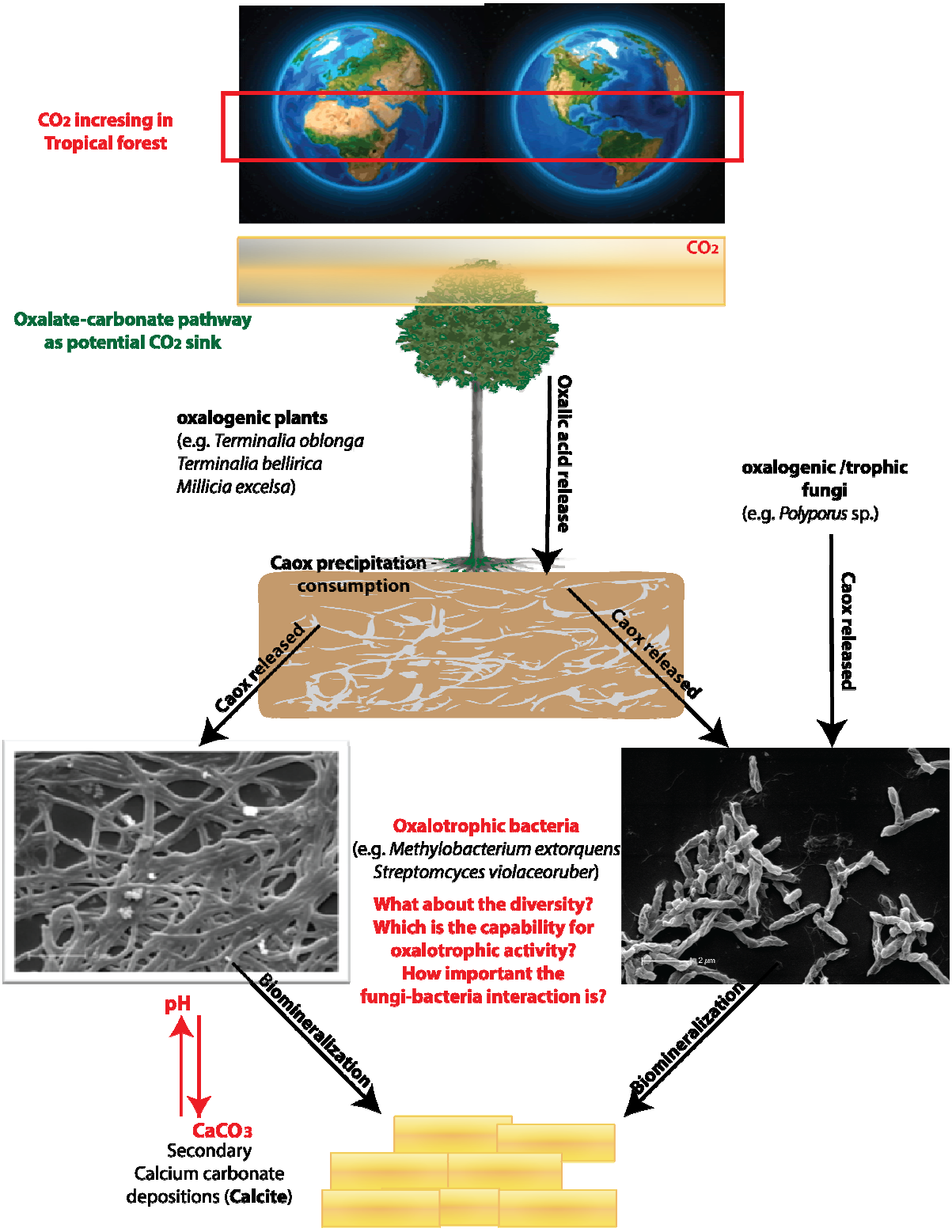

Figure 1. Sketch showing the oxalate-carbonate pathway occurring in tropical soils and the main questions taken into account in this doctoral thesis. The OCP as part of the carbon cycle occurs in many terrestrial ecosystems.

Three major biological players are involved in the oxalate-carbonate pathway: oxalogenic trees, oxalogenic (oxalotrophic) fungi and oxalotrophic bacteria (Figure 1). The systems composed by these three organisms have been observed in several sites on the planet, where they appear to lead to CO_2 storage through the accumulation of calcite. The focus of the present study is oxalotrophic bacteria, oxalotrophic activity and the interactions between soil microorganisms involved in the metabolism of oxalate.

The production of oxalic acid ($H_2C_2O_4$) (Figure 2a) and oxalate salts such as calcium oxalate (Caox, Figure 2b) is observed among a wide variety of organisms including plants, animals, fungi and bacteria present in soil (Cromack, *et al.*, 1977, Tamer, *et al.*, 2002). Oxalic acid is often accumulated as a metabolic end-product in plant cells. This can be liberated by root systems as free acids (Cailleau, *et al.*, 2005). Oxalic acid is the simplest of the dicarboxilic acids (Dijkhuizen, *et al.*, 1977). Deposition of calcium oxalate occurs in a wide range of plant taxa -215 plant families reported so far- (Nakata, 2003), and it can comprise up to 85% of the dry weight of some plants (Nakata, 2003, Franceschi & Nakata, 2005). After the death and decay of plants, oxalate is released into the soil litter where it can play important roles (e.g. in plant nutrition by increasing the availability of phosphorous and other micronutrients). Interestingly, despite oxalate relative insolubility and chemical stability, the accumulation of metal oxalates has not been observed in geological records (Schilling & Jellison, 2004). This supposes a microbiologically mediated process as the main oxalate sink in natural environments (Braissant, *et al.*, 2002). The implications of the diversity and the metabolic activity of the bacteria involved in the oxidation of oxalate are introduced in the next section.

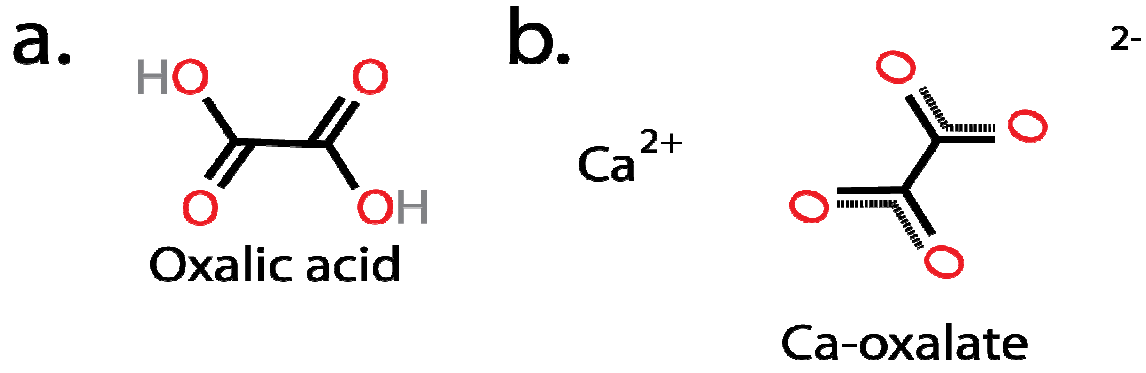

Figure 2. Molecular structure of (**a.**) the oxalic acid produced by oxalogenic plants, and (**b.**) calcium oxalate (Caox) found in litter from the oxalogenic plants in which the oxalate-carbonate pathway has been described. Calcium oxalate is a common product found in lithosphere located in litter and around oxalogenic trees. Caox is the main oxalate source to be mineralized by oxalotrophs.

c. The oxalotrophic bacterial diversity

Why is it important to study oxalotrophic bacterial diversity?

So far, there have been only few studies dealing with the diversity of oxalotrophic bacteria in temperate environments (Sahin*, et al.*, 2002, Tamer*, et al.*, 2002, Sahin*, et al.*, 2008). However, the diversity and metabolic capabilities of environmental strains from tropical soils remain unknown. Although some studies have been carried out to assess the diversity of oxalotrophic bacteria, never before, a study was performed to assess the diversity of this functional group in the context of the OCP.

Oxalotrophic bacteria do not constitute a phylogenetic group (Sahin, 2003). They are a functional group that consists of diverse taxa of microorganisms that share the physiological property of oxidizing oxalate. The fact that oxalotrophs are a functional group and do not belong to the same taxonomic group implies that their study cannot rely on traditional phylogenetic molecular markers such as the 16S rRNA gene, but rather on functional genes directly involved in the metabolism of oxalate.

A significant amount of work has been already carried out for the use of a large fragment of the *frc* gene (473 pb) as a functional marker to study bacterial diversity and oxalotrophic activity in the environment (Khammar*, et al.*, 2009). The *frc* gene codifies to formyl-CoA transferase EC: 2.8.3.16 that is implicated in the activation of the oxalate molecule to oxalyl-CoA by cycling the CoA moiety from formyl-CoA (Sidhu*, et al.*, 1997). More information on the role of the formyl-CoA transferase will be given in the next section.

A phylogenetic tree based on the *frc* sequences of model oxalotrophic bacteria and reference strains used in this study is presented in figure 3. The assessment of non-culturable oxalotrophic bacteria in soil samples, the diversity of such fraction of the oxalotrophic community in tropical soils, and the implication of such fraction in the context of the OCP will be discussed in the last chapter of this manuscript. Detailed information regarding the assessment of the diversity of oxalotrophs in tropical OCP systems is presented in chapter 4.

Besides the phylogenetic diversity of oxalotrophs involved in the OCP in tropical soils, the capability of these bacteria to consume oxalate as sole carbon and/or energy source was

unknown and their metabolic capability related with the OCP was poorly described. The current knowledge in the metabolism of oxalate is presented in the next section.

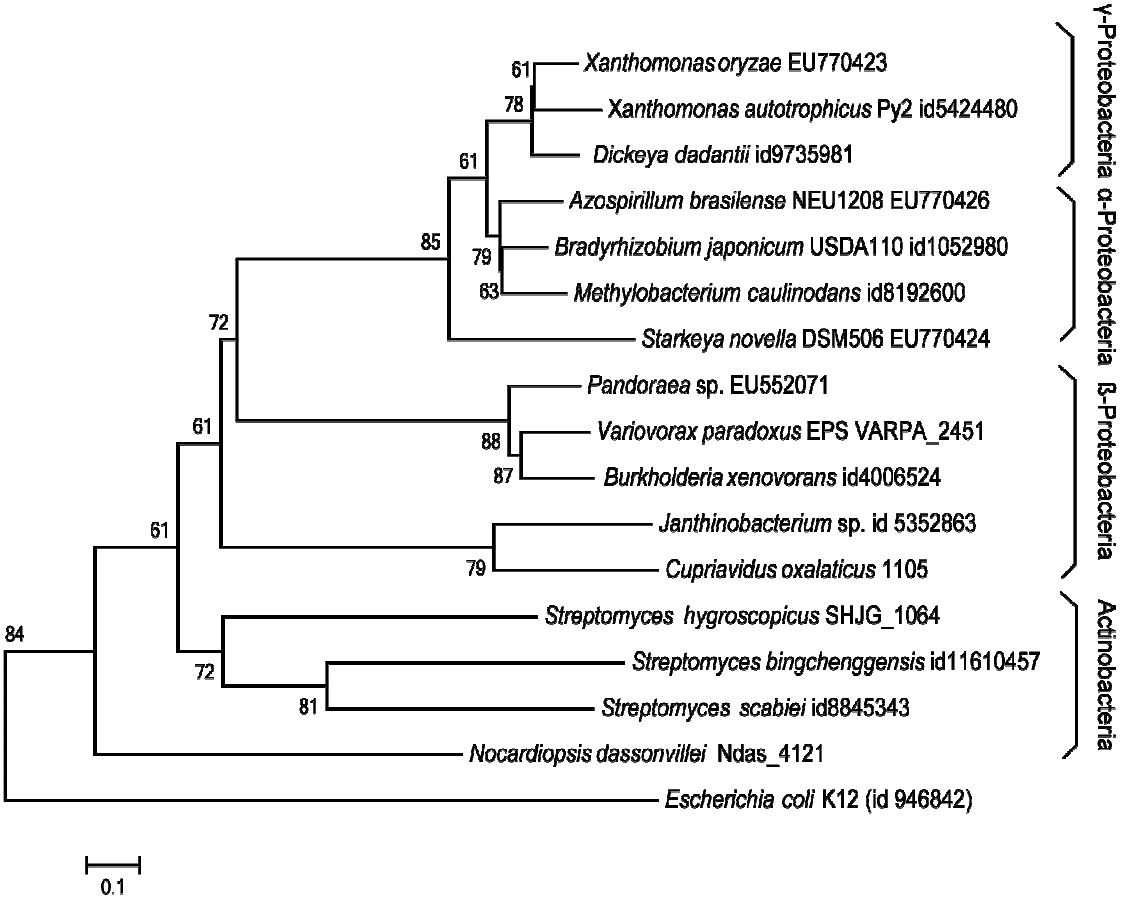

Figure 3. A phylogenetic tree based on *frc* gene sequences from model strains and those related *frc* sequences available in the GenBank database of the NCBI. Model strains used in this study were: *B. japonicum, Methylobacterium extorquens* (not showed, but related to *Methylobacterium caulinondans*), *Pandoraea* sp., *Variovorax paradoxus*, *Cupriavidus oxalaticus*, and *Streptomyces violaceoruber* (not showed, but related to *Streptomyces hygroscopicus*). The *frc* sequence from *E. coli* was used as an outgroup since this species is not able to oxidize Caox.

d. The oxalotrophic metabolism

Why to study the metabolism of oxalotrophic bacteria in tropical soils?

Bacteria using oxalate minerals, as for example calcium oxalate (Caox, Figure 2b) as source of carbon and energy are so-called oxalotrophic bacteria. Before, this group was knew as oxalate-degrading bacteria (Müller, 1950). Oxalotrophic bacteria can be considered "generalists" when they are able to metabolize many other substrates or "specialists" when they use oxalate as sole source of carbon and energy (Trinchant & Rigaud, 1996, Sahin, 2003), even if a few strains has been recognized as specialists, such in the case of *Oxalobacter formigenes*.

The study of oxalotrophic metabolism started during the first decade in the last century. For example, the strain *Methylobacterium extorquens* (before known as *Bacillus extorquens*) was recognized as degrader of sodium oxalate and oxalic acid in 1913 (Müller, 1950). Strains related to actinobacteria were as well identified to consume sodium oxalate (Müller, 1950, Khambata & Bhat, 1953). Naturally, these initial reports were merely descriptive indicating colony morphology and the degradation of oxalic acid or sodium oxalates (Müller, 1950). Nevertheless, the diversity and metabolism of oxalotrophic bacteria was getting atractive within the scientific community.

Further experiments were developed principally in the seventy's on oxalotrophic metabolism. They were focused on the understanding of the metabolic pathways involved in oxalate oxidation through bacterial metabolism of model strains, like *Cupriavidus oxalaticus*, formely *Pseudomonas oxalaticus* (Dijkhuizen*, et al.*, 1977, Friedrich*, et al.*, 1979), and *Methylobacterium extorquens* AM1 (Quayle & Keech, 1960, Quayle*, et al.*, 1961). Nowadays, *M. extorquens* AM1 is still a useful oxalotrophic model for the study of metabolic pathways (Schneider*, et al.*, 2012) and the comparison of oxalate degradation with environmental strains (Tamer*, et al.*, 2002, Sahin*, et al.*, 2008).

The studies carried out using the strain *Cupriavidus oxalaticus* (OXI) (Quayle*, et al.*, 1961) have emphasized that oxidation level of oxalate is such that incorporation of its carbon into cell constituents must involve a net reduction. Therefore, most bacteria may not use oxalate as the first choice for an energy source because due to its chemical nature and low electron yield (Dutton & Evans, 1996, Clausen*, et al.*, 2008). Besides, oxalate is a highly oxidized substrate; its

oxidation to CO_2 provides two low potential electrons that enter in the respiratory chain through formate-dehydrogenase, allowing direct reduction of NAD^+ (Blackmore & Quayle, 1968).

Interestingly, it has been observed, that thermodynamically, the oxidation of oxalate, generate a greater yield of free energy per 2 moles of e^- incorporated into the electron chain in oxalate oxidizer bacteria with -328Kj per pair e^-, compared with other carbon sources like glucose, with -239 Kj per pair e^- (Braissant, 2005). For example, the energy produced in the catabolism of Caox in *Cupriavidus oxalaticus* is used with approximately the same efficiency as in a range of other chemoorganotrophs (Dijkhuizen*, et al.*, 1977). It has been suggested that oxidation of oxalate is preceded by a rapid evolution of CO_2, suggesting that the oxidation proceeds via formate (Quayle*, et al.*, 1961). Nevertheless, this could not be the case for environmental strains. All the points mentioned above should be taken into account when oxalate metabolism is described in environmental bacteria (Chapter 4), since their preferential metabolic strategies will be reflected on the utilization of certain substrates. Several studies show that oxalate consumption rate in soils is rather high (van Hees*, et al.*, 2002). Since this consumption is mostly biological, we can assume that a rather large number of microbes do prefer oxalate over other organic compounds in the surrounding microniche.

A few approaches have been developed to measure oxalotrophic activity from environmental oxalotrophs (van Hees*, et al.*, 2002). Nonetheless, none of available methods was validated to assess the metabolic capacity of oxalotrophs *in situ*. The comparison between growth and consumptions rates of oxalate was not clearly defined, especially for environmental oxalotrophic bacteria. The fact that new metabolic pathways are associated to respiration instead of consumption or dissolution could give an idea of the efficiency of the OCP system *in situ*.

Therefore, new methods were developed in this study for measuring oxalotrophic activity to gain a deeper understanding of oxalate catabolism *in vitro* and *in situ*. The isothermal microcalorimetry assay (IMC) is a technique that allows measuring the metabolic activity of oxalotrophic bacteria and has been employed for the comparison of the catabolic performance of selected strains in different sources of oxalate (Chapter 2; Bravo*, et al.*, 2011). The application of this and others techniques will give insights on the activity of oxalotrophic bacteria isolated from various tropical soils.

The metabolic and genetic characterization of new oxalotrophic bacteria isolated from tropical soils (Chapter 4) also shed light on the catabolism of oxalate. According to our current knowledge about the oxidation of oxalate via formate, the oxidation of oxalate implied first, the activation of oxalate to oxalyl-CoA by the action of the formyl coenzyme A transferase (enzyme codified by the *frc* gene mentioned previously), and second the reduction of oxalyl-CoA to glyoxylate by the action of the enzyme oxalyl-CoA reductase. These two steps appear to be common to all oxalotrophic bacteria known so far (Schneider*, et al.*, 2012). For the assimilation of oxalate, oxalyl-CoA can be metabolized by a pathway not involving glyoxylate, which implies the action of the oxalyl coenzyme A decarboxylase (codified by the gene *oxc*). A third important common step for most oxalotrophic bacteria is the conversion of oxalate into formate by the oxalyl-CoA decarboxylase (E.C. 4.1.1.2) (Svedruzic*, et al.*, 2005).

As mentioned above, a recent study was carried out to develop primers for the amplification of the *frc* gene as a functional marker to study diversity of oxalotrophic bacteria in the environment (Khammar*, et al.*, 2009). However, in this thesis, the amplification of that gene using environmental samples and oxalotrophic isolates was only partially successfull. Therefore, in further studies, design of new molecular markers for the amplification of the *oxc* gene, other important gene for oxalotrophy should be necessary. This will allow a better assessment of the non-culturable oxalotrophic bacteria found in soil. A discussion of these results is presented in the chapter 5.

Even though the amplification of *frc* from environmental samples is a first window into the functioning of the OCP in nature, such amplification may not give enough information on the active fraction of the oxalotrophic community in soils. Therefore, a new application for studying "active" non-culturable bacteria was developed in this study (Bravo*, et al.*, 2013). The method was based on a combination of molecular-based approaches using the DGGE assay and the DNA-labeling assay with Bromodeoxyuridine (BrdU), to identify active oxalotrophic bacteria in soil microcosms. More information about the active non-culturable oxalotrophic bacteria would be presented in chapter 3.

Finally, one of the most important outcomes of this thesis is the discovery that fungi and bacteria interactions (Chapter 7) are fundamental for the oxidation of oxalate in soil. This final aspect will be developed in the next section.

e. Fungi-bacteria interactions and their role in the oxalate-carbonate pathway

What is the importance of fungi and bacteria interactions for the OCP?

A new method for the isolation of bacteria and fungi involved in the oxalate-carbonate pathway was developed (Bravo*, et al.*, 2013). These experiments allow establishing a collection of culturable oxalotrophic bacterial strains and associated fungi. This new method, called "inverted Petri dish" (Figure 4), is very important to explain the ecological relationships of fungi and bacteria and their impact in the OCP. In soils, oxalotrophic bacteria are clearly not the only player in the pathway, but one components of the system (see Figure 1). Therefore, it is very important to recognize the role of biological interactions, and not only the addition of the microbiological players (synergy) (Martin*, et al.*, 2012) or even other ecological relationships.

The study of the relationships of those microorganisms over the pathway was considered. Several key questions in this new topic have been identified. For instance, is there a synergistic or antagonist relationship between oxalogenic and oxalotrophic players? And, those interactions are important considering the real metabolic utilization of oxalate and consequent carbonate precipitation *in situ* processes?

The "inverted Petri dish method" is the first step to isolated culturable oxalotrophs and oxalogenic/oxalotrophic microorganisms to be used in further studies. Moreover, several interactions could be addressed using natural couples identified so far by this method. In the case of bacteria, this collection will be compared with the *in situ* diversity, in order to assess the representativity of the oxalotrophic microorganisms present at different sampling depths near to an oxalogenic tree. The "inverted Petri dish method" improved our knowledge in the ecology of the oxalate-carbonate pathway in tropical forest. The case of the Cameroon soil system is presented in chapter 5.

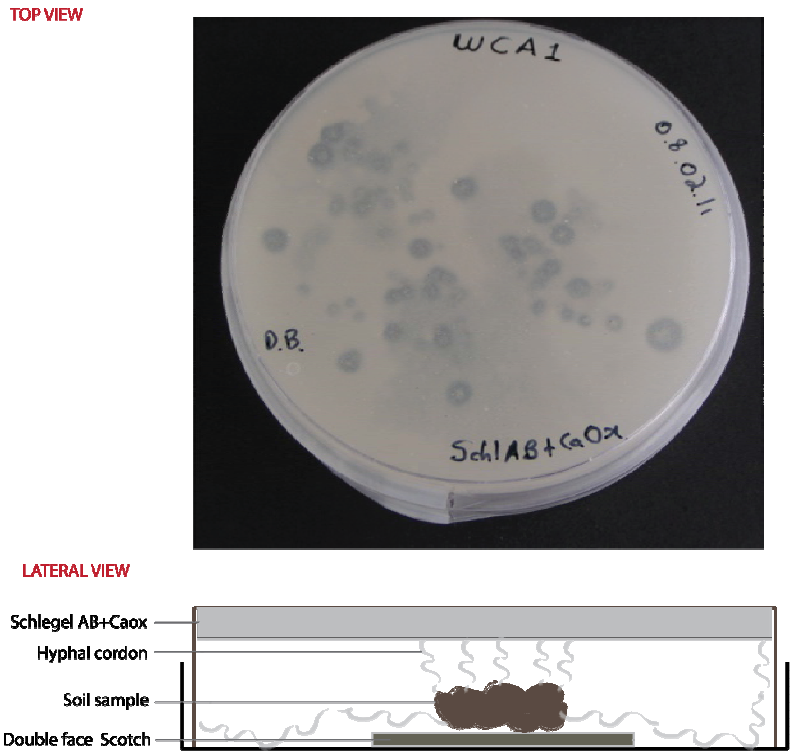

Figure 4. The inverted Petri dish method. In this method, the nutrients and the sample are opposite to the normal disposition in the Petri dish. As shown in the sketch (Lateral view), the soil sample is placed on the cover of the Petri dish while the target medium (Schlegel AB with calcium oxalate – CaOx) is placed on top of it, but separated of the sample by an air gap. As a consequence, oxalotrophic bacteria are transported to the selective medium through fungal mycelium growing from the soil to the medium. After growth of oxalotrophic bacteria, degradation halos of Caox are visual to the naked eye (Top view).

f. Aims of this research

Overall, the aims of the current research were first, to assess the diversity of oxalotrophic bacteria involved in the oxalate carbonate pathway (OCP) in tropical habitats. Second, to assess the metabolic capability of the oxalotrophs found *in situ*. For that purposes, new methodological approaches were developed in this study. On the one hand, a non-destructive analytical method based in thermodynamics and heat was designed to determine the metabolic rates of environmental oxalotrophic bacteria. On the other hand, a new BrdU labeling *frc* gene assay was developed to characterize active oxalotrophic bacteria in a microcosm environment. Moreover, isolation of culturable oxalotrophic bacteria was carried out including a characterization of selected oxalotrophs from three tropical soils using one of the new methodologies developed. Finally, a new method for recovery of fungi and oxalotrophic bacteria was developed using soil samples from the Cameroon OCP system.

This research contributes to our understanding of the role of oxalotrophic bacteria in a potential terrestrial carbon sink process based on the bacterial mineralization of oxalate into carbonate. The first two chapters describe the two techniques developed to study the oxalotrophic metabolism, and the diversity of active oxalotrophic bacteria *in situ*. The two last sections are related to the isolation and characterization of oxalotrophic bacteria, and the new method of Petri dish to recover couples of fungi and bacteria involved in the OCP. Finally a global discussion, including a comparison of the study sites, concludes this thesis.

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Chapter 2. Use of an isothermal microcalorimetry assay to characterize microbial oxalotrophic activity

Daniel Bravo, Olivier Braissant, Anna Solokhina, Martin Clerc, Alma U. Daniels, Eric Verrecchia, Pilar Junier. 2011. *FEMS Microbiology Ecology* **78**: 266-274

How to assess the metabolic capability of oxalotrophic bacteria?

Chapter summary

Due to the fact that our knowledge about oxalotrophic activity in bacteria was incipient, it was necessary to develop new tools to better understand microbial oxalotrophy. Therefore, this chapter introduces the isothermal microcalorimetry as a new technique to measure metabolic rates of oxalotrophic bacteria. Isothermal microcalorimetry is an analytical technique based on heat production, and thermodynamics, to detect even low signals of heat produced by bacterial growth or metabolic activity. The high sensitivity of heat detection and the use of techniques *in statu nascendi* for microbial ecology, such as IMC, are highlighted through this chapter.

Abstract

Isothermal microcalorimetry (IMC) has been used in the past to monitor metabolic activities in living systems. A few studies have used it on ecological research. In this study, IMC was used to monitor oxalotrophic activity, a wide spread bacterial metabolism found in the environment, and particularly in soils. Six model strains inoculated in solid angle media with K-oxalate as the sole carbon source. *Cupriavidus oxalaticus*, *Cupriavidus necator*, and *Streptomyces violaceoruber* presented the highest activity (91, 40, and 55 microwatts, respectively) and a maximum growth rate (μmax [h^{-1}]) of 0.264, 0.185, and 0.199, respectively, among the strains tested. These three strains were selected to test the incidence of different oxalate sources (Ca, Cu, and Fe-oxalate salts) in the metabolic activity. The highest activity was obtained in Ca-oxalate for *C. oxalaticus*. Similar experiments were carried out with a model soil to test if this approach can be used to measure oxalotrophic activity in field samples. Although measuring oxalotrophic activity in a soil was challenging, there was a clear effect of the amendment with oxalate on the metabolic activity measured in soil. The correlation between heat flow and growth suggests that IMC analysis is a powerful method to monitor bacterial oxalotrophic activity.

a. Introduction

Oxalic acid ($H_2C_2O_4$) and oxalate minerals are widely distributed among plants, animals, fungi, and bacteria present in soils (Tamer*, et al.*, 2002). Oxalic acid is often accumulated as a metabolic end product in plant tissues or is released by root systems as a free organic acid or mineral salts such as calcium, iron, or magnesium oxalate (Cailleau*, et al.*, 2005). The oxalate released in soils can play important roles, e.g. by increasing the availability of phosphorous and other micronutrients for plant uptake (Franceschi & Nakata, 2005). The release of calcium oxalate has also been involved in the formation of calcite ($CaCO_3$) deposits in otherwise acidic carbonate-free soils (Braissant*, et al.*, 2004, Cailleau*, et al.*, 2004, Dupraz*, et al.*, 2009). This process, also called the oxalate-carbonate pathway (Cromack*, et al.*, 1977, Verrecchia, 1990, Verrecchia*, et al.*, 2006), relies on the biological degradation of oxalate and has been pointed out as an underestimated long-term carbon sequestration mechanism (Braissant*, et al.*, 2002). The biological degradation of oxalate is in agreement with the fact that accumulation of metal oxalates has not been observed in geological records (Schilling & Jellison, 2004, Verrecchia*, et al.*, 2006), except in some very specific settings such as hydrothermal springs (Hofmann & Bernasconi, 1998) and septarian concretions (Hyde & Landy, 1966). Normally, a microbiologically mediated process is assumed to be the main oxalate degradation process in natural environments, leading to the precipitation of calcium carbonate or other forms of carbonate depending on the oxalate mineral sources (Robbel & Kutzner, 1973).

Bacteria using oxalate as a source of carbon and energy are called "oxalotrophic bacteria" (Sahin, 2003). Oxalatrophic bacteria can be considered "generalists" when they are able to metabolize other substrates, or "specialists" when they use oxalate as their sole carbon and energy sources (Trinchant & Rigaud, 1996). There are still many questions regarding the specific metabolic pathways involved in the use of oxalate. The first metabolic studies on oxalate-consuming bacteria started a long time ago (Quayle & Keech, 1959, Blackmore & Quayle, 1968, Dijkhuizen*, et al.*, 1977, Aragno & Schlegel, 1981). For its use as carbon and energy sources, in *Cupriavidus oxalaticus* (Vandamme & Coenye, 2004) oxalate is activated to oxalyl-CoA before a part of it is oxidized to CO_2 (energy generation) and the remaining part is reduced to glyoxylate for biosynthesis (Jayasuriya, 1955, Blackmore & Quayle, 1970). Biosynthesis is thought to occur either via the serine or the glycolate pathways (Tamer & Aragno, 1980, Sahin, 2003). Due to the fact that oxalate is a highly oxidized substrate, bacterial growth yield is low (about 2.5 g mol^{-1}) (Braissant*, et al.*, 2002). In addition to the direct assimilation of oxalate, this organic salt has been

implicated in pH regulation and aluminum detoxification in non-oxalotrophic bacteria (Friedrich*, et al.*, 1979).

Previous studies have described various methods for measuring oxalotrophic activity. These methods imply either disruption of the cells allowing measurements of enzymatic activity (Quayle & Keech, 1960, Quayle, 1963, Milardović*, et al.*, 2000); or colorimetric assays in order to measure pH changes linked to oxalate consumption (Cromack*, et al.*, 1977). More recently, a new molecular marker has been identified as specific to oxalotrophic bacteria (gene *frc* codifying for the enzyme formyl-CoA transferase) (Khammar*, et al.*, 2009). This opens up the possibility of detecting oxalotrophic activity through transcriptomic studies. However, there are some oxalotrophic bacteria that do not have a functional *frc* gene but are able to grow *in vitro* using selective media with calcium oxalate (false negatives), or bacteria that contain the *frc* gene but do not use oxalate as a carbon or energy source (false positives; e.g. *Escherichia coli* K12; Bravo et al., unpublished results). Therefore, alternative approaches are needed to estimate oxalotrophic activity and to improve our comprehension of oxalate metabolism by bacteria. Isothermal calorimetry could fill such a gap by allowing measuring oxalotrophic activity in real-time over a wide variety of conditions.

The term isothermal microcalorimetry (IMC) is commonly used to refer to the measurement of heat production in the microwatt range under essentially isothermal conditions (Wadsö & Goldberg, 2001). Several types of isothermal microcalorimeters exist, making possible nearly isothermal conditions through phase transition, power compensation, or heat conduction. Nowadays, heat conduction microcalorimeters (also called heat flux microcalorimeters) are the most commonly used type (van Herwaarden, 2000, Braissant*, et al.*, 2010). Isothermal titration calorimetry (ITC) – a particular application of IMC – has been widely used to characterize binding affinities of various ligands to protein and macromolecules (Cooper, 2003). In contrast to ITC, IMC has been less commonly used in biological sciences compared to other areas. However, isothermal microcalorimetry (IMC) can be used to measure oxic and anoxic metabolisms, with reproducibility and long-term baseline stability, and it is therefore pertinent for measuring microbial activity in soils (Vor*, et al.*, 2002, Rong*, et al.*, 2007, Wadsö, 2009). In this way, every process in soil can be recorded as a specific metabolic activity in function of heat production. The importance of calorimetric analysis is supported by the fact that results can be expressed as "life intensity" or "biological response" (Mortensen*, et al.*, 1973). In addition, results can be expressed in terms of thermodynamic or kinetic properties of bacterial activity and are directly related with other experimental conventional techniques (see review in Braissant*, et al.*,

2010). Despite its potential for measuring exothermic reactions associated with the consumption of oxalate, at the moment, there are no precedents on the use of IMC to study oxalotrophic activity, and in particular in soils.

In this study, the growth of oxalotrophic bacteria is characterized for the first time using IMC. The aim of this study is to describe the oxalotrophic activity of model strains and in a model soil. Three different types of experiments were performed. First, several model oxalotrophic strains were grown in potassium oxalate in order to compare their respective heat release during oxalate consumption. Second, a group of selected strains was used to compare oxalotrophic activity in the presence of different oxalate sources (K, Ca, Cu, and Fe). Finally, a model soil was used to test the potential of IMC to measure oxalotrophic activity in soils. In this context, the present study contributes to the understanding of microbial ecology of oxalotrophic activity in soil bacteria.

b. Materials and Methods

i. Bacterial strains and growth conditions

Four generalist strains were used to test the pertinence of IMC to measure oxalotrophic activity. *Methylobacterium extorquens, Cupriavidus oxalaticus, Streptomyces violaceoruber*, and *Cupriavidus necator* have been reported as oxalotrophic bacteria (Tamer*, et al.*, 2002, Sahin, 2003). In addition, *Escherichia coli* was used as a negative control. *E. coli* has been reported as a non-oxalate oxidizing bacteria (Turroni*, et al.*, 2007). The strain BV1M3 corresponds to a *Streptomyces* sp. and was isolated from a tropical soil in Bolivia. This soil was collected near the oxalogenic tree *Terminalia oblonga*. All cultures were maintained in nutrient broth or nutrient agar (NB/NA) media (Difco) for regular transfers. All the strains used in this study are summarized in Table 1.

Table 1. Oxalotrophic strains used in study. All these strains are deposited in the culture collection of the Laboratory of Microbiology, University of Neuchâtel (LAMUN).

Strain	Collection number	Strain number
Escherichia coli K12	NEU 1007	K12
Methylobacterium extorquens	NEU 44	TA3
Cupriavidus oxalaticus	NEU 1047	OX 1
Streptomyces violaceoruber	NEU 1225	-
Cupriavidus necator	NEU 2073	NS2
Streptomyces sp. BV1m3	BV1m3	BV3

ii. Growth conditions for calorimetric assays in pure cultures

For growth in the microcalorimeter, all strains with the exception of *E. coli*, were preincubated on solid Angle media (Angle*, et al.*, 1991) supplemented with 4 g L^{-1} calcium oxalate as carbon source. *E. coli* was pre-inoculated on Nutrient agar (NA). Replicates cultures for all strains were performed in 4 mL microcalorimetric ampoules filled with 2 mL of slanted solid Angle's medium to which 4 g L^{-1} potassium oxalate (pH = 7.0) was added. Inoculation was performed with an inoculation loop, ensuring that bacteria would grow as a loan. Previous studies have shown that decreasing inoculum concentration resulted in a lower maximum activity (Braissant*, et al.*, 2010, Braissant*, et al.*, 2010). The measurements of oxalotrophic activity were performed in an isothermal heat conduction microcalorimeter (TAM III, Waters/TA Instruments, Delaware, USA) equipped with 48 channels. The temperature of the microcalorimeter thermostat was set for growth of environmental bacteria at 25°C. After stable temperature conditions were obtained, each measuring channel was calibrated using a built-in electrical heater of known power. All the individual microcalorimeters (i.e., measuring channels) also have a built-in aluminum reference with a heat capacity and conductivity approximately equal to that of a 4 mL glass ampoule containing 3 mL of water. This reduces equilibration time and improves the stability of the heat-flow rate measurements. The baseline was obtained from ampoules containing sterile medium only. Therefore, any heat production by the media (i.e., chemical heat) could be subtracted from the signal in the ampoules inoculated with the strains. However, such subtraction was not necessary since measurement using sterile media remained within the background noise level. The measurements obtained in the microcalorimeter were recorded after a 2-step thermal equilibration procedure recommended by the manufacturer that lasted 1 hour. Briefly, during the first 15 minutes, the samples were placed in the equilibration position to achieve preliminary thermal equilibration. Samples were then placed in the measuring position. However, 45 additional minutes were necessary to achieve fine thermal equilibration and start measurements.

Three strains were selected for the second experiment. The strains were inoculated in an inclined Angle agar medium, supplemented with different insoluble metal-oxalate sources. Calcium, copper, and iron oxalate were selected because they are representative sources available in a soil (Cromack*, et al.*, 1977). Copper oxalate was produced following the protocol by David (1960) from copper sulfate and oxalic acid. Iron oxalate was obtained using a protocol developed by the Académie de Bordeaux. Briefly, ferrous ammonium sulfate was dissolved in acidified water at 40°C (4% 2M sulfuric acid) and mixed with a solution of oxalic acid. Copper and iron oxalates were prepared at the Laboratory of Mycology, University of Neuchâtel. The sources were tested

to be the sole carbon source at 4 g L^{-1}. The isothermal microcalorimeter was filled with replicates of each carbon source per strain. The baseline was obtained from ampoules with sterile medium. The measurements obtained in the microcalorimeter were recorded after the manufacturer recommended a 2-step thermal equilibration procedure that lasted 1 hour as well.

iii. Microcalorimetric assay of soil

The soil selected as a model for the assays was used in previous studies and its physicochemical parameters have been characterized extensively (Milleret*, et al.*, 2009). The soil corresponds to the organo-mineral horizon of an Anthrosol collected at the botanical garden of Neuchâtel (Switzerland). The soil is a carbonated loamy soil (45.3% sand, 28.0% silt and 26.7% clay), containing 20.7% (w/w) carbonates, 2.0% (w/w) total organic carbon and having a pH_{KCl} of 7.8. The cation exchange capacity was 21.3 cmol $_c$ kg^{-1}. Soil was sieved in the laboratory to remove root fragments and large particles. Four treatments were applied to the soil i) no additions; ii) spiked with 10^9 CFU mL^{-1} of *C. oxalaticus*; iii) addition of Angle liquid medium (800 µL) with 4 g L^{-1} potassium oxalate; and iv) spiked with 10^9 CFU mL^{-1} of *C. oxalaticus* and Angle liquid medium (800 µL) with 4 g L^{-1} potassium oxalate. Two grams of soil were added in each 4 mL microcalorimetric ampoule. For the treatment without additions (i), the soil was homogeneously humidified with 800 µL of liquid sterile angle media without a carbon source. All treatments were run at 25°C. As for the cultures on solid media, the measurements obtained in the microcalorimeter were recorded after the manufacturer recommended a 2-step thermal equilibration procedure described above.

iv. Data analysis

Analysis of the heat flow (thermograms) was used for calculating the kinetic parameters. Since $1\mu W = 1\mu J \bullet s^{-1}$, the maximum heat flow (Figure 1A) was used to calculate the maximum oxalotrophic activity assuming the following reaction $2H_2C_2O_4 + O_2 \rightarrow 4CO_2 + 2H_2O$ with a reaction enthalpy of $\Delta H°'$ of -499KJ•$mole^{-1}$ (standard enthalpies of formations of reactants and products were obtained from (Dean, 1999). Such calculation assumes that the heat produced by biomass formation is ignored since biomass yield on oxalate is very low. However, it must be noted that models exist to estimate the amount of heat released during biomass production (Heijnen*, et al.*, 1992). The heat over time curve was obtained by integrating the heat flow data (Figure 1B). Using the heat over time curve, the net growth rate was calculated by fitting an exponential model ($Q_t = Q_0 \bullet e^{\mu t,}$ where Q represents the heat) over the exponential part of the curve. Similarly, the maximum growth rate was calculated by fitting the modified Richard's

equation (Zwietering, *et al.*, 1990) to the complete heat over time curve. For every strain, these calculations where performed over three to six replicates allowing the calculations of mean and standard deviation. The thermograms of each strain were analyzed in terms of thermodynamic consumption of oxalate in function of oxalotrophic activity and oxalate source.

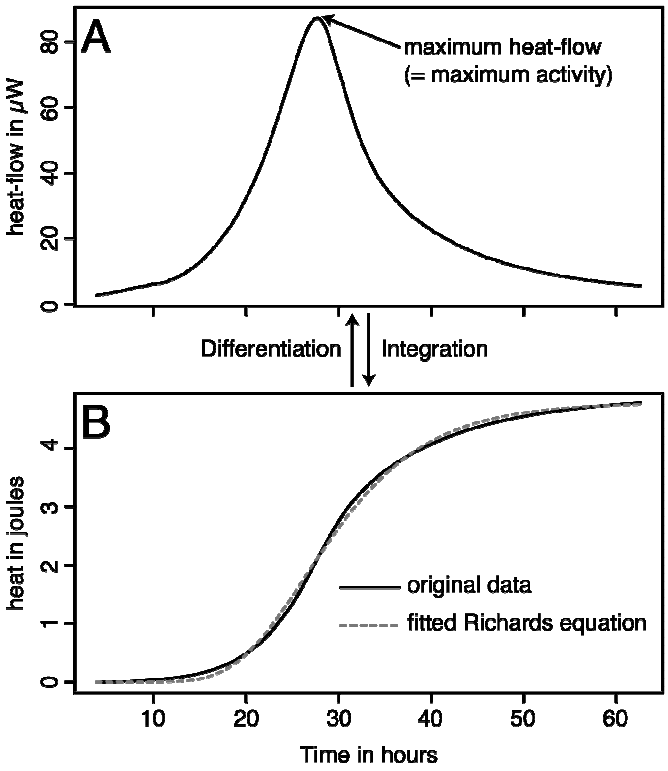

Figure 1. Sketch showing the relationship between actual calorimetric measurements (heat- flow; **A**) which corresponds to the microbial activity and the evolution of heat (**B**) which is a proxy for products resulting from oxalotrophic activity (i.e., biomass or CO_2 for example). The maximum heat flow (arrow) is directly proportional to the maximum oxalotrophic activity of the strain.

To calculate the accuracy of IMC for measuring oxalotrophic activity, degradation rates of oxalate calculated by IMC were compared with degradation obtained by HPLC using the same strains and medium (in this case in liquid). The chromatograms were obtained using a HPLC 110 (Agilent Technologies) with a diode array detector (DAD - 210nm UV) and a column RPC18. The method carried out was according to previous studies for oxalogenic fungi (Schilling & Jellison, 2004), with the following modifications for oxalotrophic bacteria: the samples were centrifuged at 10.000 x g for 2 minutes. Five-hundred µL from the supernatant were dissolved in 800 µL of 20mM H_2SO_4 HPLC grade. The solution was mixed vigorously by pulsed vortexing for 20 seconds. The samples were incubated for 2 h at room temperature. Before HPLC analysis, the

samples were filtered with Whatman membrane cellulose filters of 0.45 μm pore size in 1.5 mL vials closed with septum caps.

c. Results

i. Oxalotrophic activity measured by IMC in pure cultures

The aim of this first experiment was to compare oxalotrophic activity between strains and select those with higher efficiency on the degradation of the carbon source to be tested with alternative metal-oxalate sources. Potassium oxalate was selected as a substrate to measure oxalotrophic activity in four known oxalate-consuming bacteria, and one environmental strain. In addition, *E. coli* was used as negative control. Representative thermograms obtained for each one of the strains are shown in Figure 2. In the case of the negative control (*E. coli*), even after 280 hours of incubation at 25°C, no change in the base line signal was observed. In contrast, in all the other strains (known to consume oxalate), a clear peak of activity was observed. For *C. oxalaticus, M. extorquens*, and *C. necator*, maximum heat flow was observed before 50 hours of incubation. In the two strains of actinobacteria (*S. violaceoruber* and *Streptomyces* sp. BV1M3), the maximum heat flow was recorded considerably later between 100 and 200 hours.

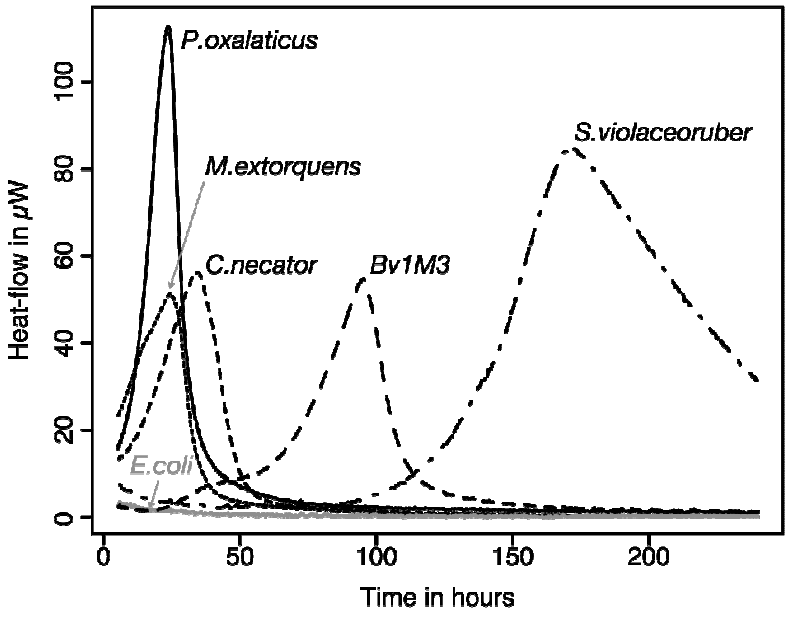

Figure 2. Representative thermograms recorded for *Cupriavidus oxalaticus*, *Methylobacterium extorquens*, *Cupriavidus necator, Streptomyces violaceoruber, Sptreptomyces* sp. BV1M3 and *Escherichia coli* inoculated in Angle medium with potassium oxalate as the sole carbon source.

The parameters of biological activity derived from the heat flow (Table 2) show that *C. oxalaticus* has the highest activity on potassium oxalate (measured as consumption rate), followed by *C. necator*, and *M. extorquens*. In all the cases there was a linear correlation between the maximum oxalate consumption rate and the net (Figure 3A) and maximum (Figure 3B) growth rates. Values slightly deviating from the linear trend might be due to maintenance effects for the different strains.

Table 2. Kinetic parameters obtained after analysis of heat flow thermograms obtained in Angle medium with potassium oxalate (**A**) and nutrient agar (**B**). To convert the maximum heat flow into the maximum consumption rate, the following enthalpy of reaction value was used: $\Delta H°'$ of -499KJ•mole^{-1}. n= number of replicates; N.D.= not determined; N.A.= not applicable.

A.

Name	μ	μ max	max heat flow	consumption rate	n
	h^{-1}	h^{-1}	mW	μMoles.h^{-1}	
Cupriavidus oxalaticus	0.14 ± 0.02	0.26 ± 0.04	0.09 ± 0.01	0.66 ± 0.10	6
Methylobacterium extorquens	0.07 ± 0.02	0.15 ± 0.02	0.04 ± 0.01	0.32 ± 0.06	5
Streptomyces violaceoruber	0.05 ± 0.02	0.19 ± 0.09	0.04 ± 0.03	0.29 ± 0.20	5
Cupriavidus necator	0.06 ± 0.02	0.20 ± 0.03	0.05 ± 0.01	0.40 ± 0.07	6
Streptomyces sp. BV1M3	0.03 ± 0.02	0.11 ± 0.05	0.04 ± 0.02	0.26 ± 0.13	3
Escherichia coli K12	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	3

B.

Name	μ	μ max	max heat flow	consumption rate	n
	h^{-1}	h^{-1}	mW	μMoles.h^{-1}	
Cupriavidus oxalaticus	0.23 ± 0.05	0.92 ± 0.20	0.24 ± 0.04	N.A.	3
Methylobacterium extorquens	0.03 ± 0.00	0.22 ± 0.04	0.06 ± 0.01	N.A.	3
Streptomyces violaceoruber	0.07 ± 0.04	0.52 ± 0.17	0.13 ± 0.04	N.A.	3
Cupriavidus necator	0.03 ± 0.00	0.16 ± 0.03	0.05 ± 0.01	N.A.	3
Streptomyces sp. BV1M3	N.D.	N.D.	N.D.	N.A.	N.A.
Escherichia coli K12	0.16 ± 0.08	1.01 ± 0.10	0.21 ± 0.04	N.A.	3

When the same analysis was carried out in data obtained from growth in nutrient agar (Table 2B), both *E. coli* and *C. oxalaticus* showed higher maximum heat flow and growth than the other strains. For generalist strains as *E. coli* and *C. oxalaticus*, more nutrients represent more activity and a higher growth rate. Others strains do not benefit from additional nutrients and no increase of growth rate is observed. This is the case of *C. necator* and *M. extorquens,* which are specialized in C1 and C2 compounds. In nature they play key roles in the global cycling of methane, methanol, halogenated methanes, methylated sulfur compounds and organic acids in litter (Guo & Lidstrom, 2008, Toyota*, et al.*, 2008).

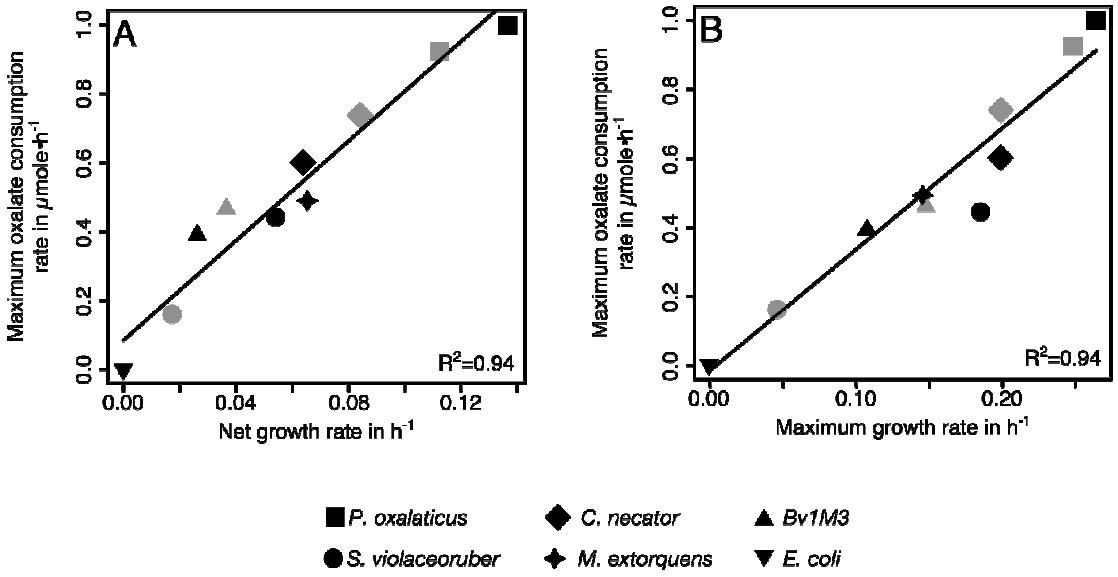

Figure 3. Data showing the correlation between the net growth rate (μ - panel **A**), maximum growth rate (μmax – panel **B**) and the maximum oxalate consumption rate for the 6 strains tested. The black symbols are for data obtained using potassium oxalate and the grey symbols are for data obtained using calcium oxalate. Correlation coefficient is indicated at the bottom of each panel.

ii. Comparison of different metal-oxalate as carbon sources

C. oxalaticus, C. necator, *S. violaceoruber*, and *Streptomyces* sp. BV1M3 were selected to test the effect of different metal-oxalates as carbon sources on growth and metabolic activity. In addition to potassium, calcium, copper and iron-oxalate were assayed. Figure 4 shows the results obtained for *C. oxalaticus* in the different oxalate sources. Heat flow in potassium and calcium oxalate showed a clear peak corresponding to the activity in these substrates. This was also the case for *S. violaceoruber*, and *Streptomyces sp.* BV1M3 in the same substrates (data not shown). For copper and iron-oxalate, the heat flow was undistinguishable from the baseline (Figure 4), and this was the case for all four strains (data not shown). These results indicate that these strains were unable to use pure copper and iron-oxalate as substrates for growth.

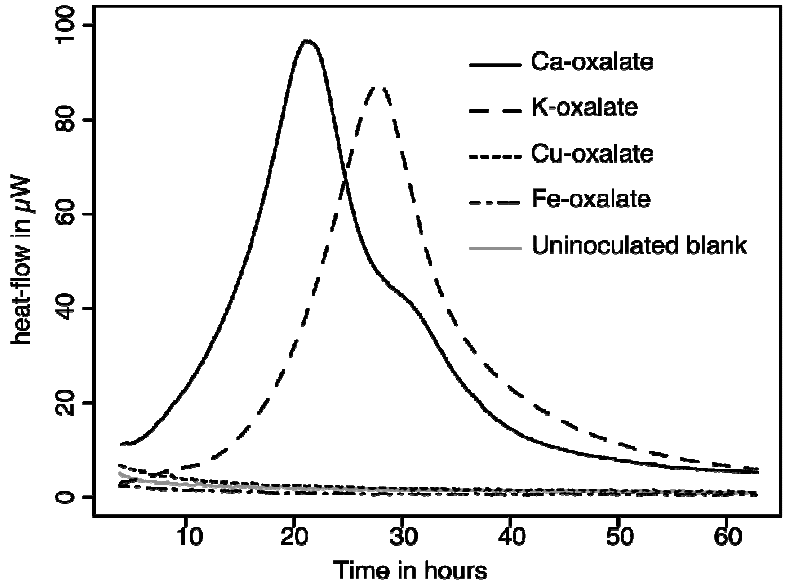

Figure 4. Thermogram curves for *Cupriavidus oxalaticus* in four metal-oxalate substrates. Data from Cu- and Fe-oxalate are indistinguishable from the baseline. The IMC method was able to distinguish differences in metal-oxalate source consumption.

The biological activity derived from the heat flow for calcium oxalate (Table 3) produce very similar results to those obtained in potassium-oxalate. Once again, the highest activity was observed in *C. oxalaticus*. Interestingly, for *C. necator* and *Streptomyces* sp. BV1M3 consumption rates on calcium oxalate were about 20% higher than the observed in potassium oxalate.

Table 3. Kinetic parameters obtained after analysis of heat flow thermograms obtained in Angle medium with calcium oxalate. To convert the maximum heat flow into the maximum consumption rate, the following enthalpy of reaction value was used: ΔH°' of -499KJ•mole^{-1}.

Name	µ	µ max	max heat flow	consumption rate	n
	h^{-1}	h^{-1}	mW	µMoles.h^{-1}	
Cupriavidus oxalaticus	0.11 ± 0.02	0.25 ± 0.03	0.08 ± 0.01	0.61 ± 0.09	3
Streptomyces violaceoruber	0.02 ± 0.00	0.05 ± 0.01	0.01 ± 0.00	0.11 ± 0.02	3
Cupriavidus necator	0.08 ± 0.00	0.20 ± 0.03	0.07 ± 0.01	0.49 ± 0.07	3
Streptomyces sp. BV1M3	0.04 ± 0.00	0.15 ± 0.02	0.04 ± 0.01	0.31 ± 0.04	3

Although the cultures were performed in liquid media, the consumption rate for calcium-oxalate determined by HPLC were closely similar to those obtained by IMC. For example, the consumption rate for *C. oxalaticus* determined using HPLC measure was 5.5μM.h^{-1}.mL^{-1}, and with microcalorimetry the consumption rate was 3.3μM.h^{-1}.mL^{-1}. These results allow the elucidation of the sensibility and power resolution of the IMC technique to measure oxalotrophy.

iii. Oxalotrophic activity measured in soil

The possibility of using IMC to measure the potential oxalotrophic activity directly in soil samples was assayed as well. Although four treatments were performed in soil (see methods), the results were grouped according only to the addition or not of potassium oxalate. The amendment with *C. oxalaticus* did not have an effect on the activity. In contrast, the addition of potassium oxalate had an effect on the heat produced in soil (Figure 5A), compared to the unamended soil. The difference in the total heat released in the presence and absence of oxalate was equivalent to the heat produced by a culture with *C. oxalaticus* growing in the presence of the same amount of oxalate. This suggests that the community in the soil indeed used the oxalate added to the soil. The heat flow resulting from comparing the data obtained for the difference in activity between amended and unamended soil (Figure 5B) showed two different activity peaks, the first one at about 50 hours, and the second one closer to 200 hours of incubation.

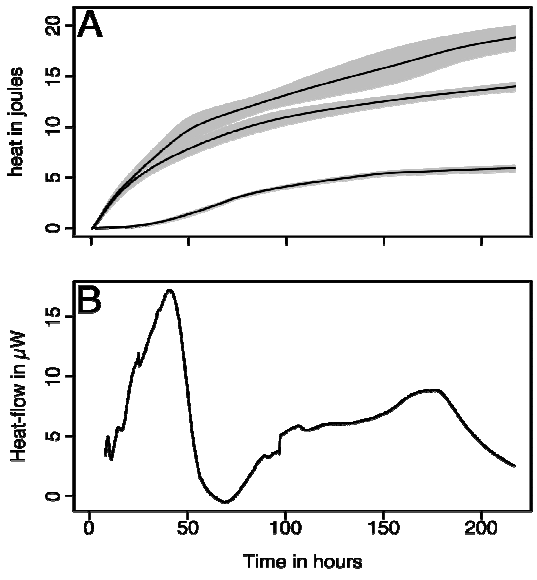

Figure 5. Heat production over time in samples from soil incubated in the presence and absence of potassium oxalate (**A**). The heat produced by *Cupriavidus oxalaticus* is presented as a comparison. Gray areas correspond to the standard deviation. The differential of the difference between the curves with and without oxalate are presented in panel B. In panel **B** two peaks of activity were observed in the presence of oxalate most likely corresponding to oxalotrophic activity.

d. Discussion

Isothermal microcalorimetry was a useful method to identify oxalotrophic activity in pure cultures. Characteristic curves of heat flow were related to the oxalotrophic activity in the five oxalate-consuming strains. Differences were already clearly visible in the raw heat flow data (maximum heat flow for example), which could be compared between the strains. In addition, other biological parameters were obtained from these data. The biological activity measured as heat release could be analyzed to obtain growth parameters in potassium and calcium oxalate substrates (Figure 2). In addition, the maximum heat flow can be related with the maximum degradation rates of oxalate. The maximum degradation rates can be compared for the different strains and oxalate salts. This is certainly an important feature when new strains are being screened for their metabolic capabilities, since it offers a quantitative mean of comparison among different substrates. In addition, IMC offers the opportunity to work with solid-state medium and relatively insoluble substrates, which are extremely difficult to approach with other conventional microbiological techniques.

Biological activity measured by IMC can also contribute to understand the metabolism of an organism. For example, *E. coli* is known to contain a copy of a gene homolog to *frc,* coding for the formyl-CoA transferase (Gruez*, et al.*, 2004), which mediates the metabolism of oxalate in *C. oxalaticus* and the anaerobic bacteria *Oxalobacter formigenes* (Sidhu*, et al.*, 1997). In addition to this, experiments carried out with the purified protein homolog to *frc* show that it can actually catalyze the synthesis of oxalyl-CoA from formyl-CoA and oxalate, as inferred based only on the genome annotation (Toyota*, et al.*, 2008). With the identification of a formyl-CoA/oxalate CoA transferase, a question that arises is the extent and importance of oxalate-related metabolism in *E. coli*. Although *E. coli* has been implicated in the biomineralization processes leading to the formation of calcium oxalate crystals (Chen*, et al.*, 2007), growth measurements in anaerobic media containing 5 mM of sodium oxalate suggest that *E. coli* does not degrade this compound (Turroni*, et al.*, 2007). The IMC experiments performed for degradation and consume rates of oxalate with in the presence of *E. coli* produced consistent results. For this strain no oxalotrophic activity in function of heat flow signal was found. After an equilibration period, no heat production was observed during the total incubation time, indicating that *E. coli* is unable to use aerobically oxalate as energy or carbon source, at least under the concentrations used (approximately 20 mM). In *E coli* the absence of growth was confirmed in the vials after the experiment. The results obtained in the presence of potassium oxalate were clearly due to the

carbon source selected, since considerable growth was observed for the experiments carried out in nutrient broth (μmax = 1.01 ± 0.09 h^{-1} and max. heat flow = 0.20 ± 0.04 mW). These results, the absence of growth in poor medium and active growth in a complex medium, appear to confirm previous observations indicating that *E. coli* needs enriched conditions to growth and develop (Boe & Lovrien, 1990).

The effect of different oxalate sources on metabolic activity could also be measured by IMC. The results showed that even though copper and iron oxalates are widespread in natural soils (Cromack*, et al.*, 1977), they are poor substrates for bacterial growth, at least for the strains tested. This is in agreement with previous statements indicating that different metals might be toxic for oxalotrophic bacteria (Sahin*, et al.*, 2002). In our study these metals were released directly from the oxalate salts and the maximum concentration of [Cu^{2+}] and [Fe^{2+}] ions in the media could be estimated at $1.73x10^{-4}$ mol.L^{-1} and $4.4x10^{-4}$ mol.L^{-1} (solubility values), respectively. On the other hand, potassium and calcium oxalate were consumed with similar rates for most of the strains tested. The only exception was *S. violaceoruber* that showed a 2-fold decrease.

In the experiments with soil the most influential factor affecting the evolution of heat was the addition of potassium oxalate (Figure 5A). Interestingly, the difference between the curves with and without potassium oxalate was almost the same as the difference between the baseline and *C. oxalaticus* heat over time curve. Since the heat difference caused by the addition of oxalate (independently of the amendment with bacteria) is equal to the heat released by *C. oxalaticus*, this suggests that soil activity is independent of the addition of bacteria. This result reflects a clear response to oxalate that most likely is a function of the potential oxalotrophic activity in soil. The low impact of amendments with *C. oxalaticus* probably is likely to occur because of the lesser importance of populations from this strain relative to global metabolic activity from autochthonous soil populations. It is important to signal that the composition and nature of the oxalotrophic communities in soil was not the focus of this study, but rather a "proof of concept" for the utilization of IMC directly on soil samples.

In the differential heat flow between soil with or without oxalate, two peaks of activity (also independent of the addition of bacteria) were observed (Figure 5B). The first peak corresponds most likely to direct oxalate consumption, as potassium oxalate will be a major carbon source added to the soil. The second peak could represent either the use of oxalate by bacteria with slower growth rates (e.g. *Streptomyces*-like) or the consumption of a less accessible pool of oxalate. In fact, in unsaturated porous media such as soils, food, or leaves, the distribution of

environmental factors is very patchy and limits both bacterial dispersion and access to the substrate (Zhou*, et al.*, 2002, Zhou*, et al.*, 2004, Wang & Or, 2010). Alternatively, the consumption of a product derived from the metabolism of oxalate could also explain the appearance of a second activity peak. Several intermediate metabolic products have been identified from the metabolism of oxalate, such as CO_2, formate or malate (Quayle*, et al.*, 1961). However, both the variability of microbial communities' access to oxalate at different rates and the impact of metabolic end-products need to be assessed in future experiments.

e. Acknowledgments

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Chapter 3. Identification of active oxalotrophic bacteria by BrdU DNA-labeling in a microcosm soil experiments

Daniel Bravo, Gaëtan Martin, Maude M. David, Guillaume Cailleau, Eric Verrecchia, Pilar Junier. 2013. *FEMS Microbiology Letters*. **In Press**

What is the active fraction of oxalotrophic community involved in the oxalate-carbonate pathway in soils?

Chapter summary

The *frc* gene is a useful tool to characterize oxalotrophic non-culturable bacteria. Nevertheless both culturable and non-culturable techniques do not give information about the active oxalotrophs (active players) that carry out the oxidation of oxalate in tropical soils. To answer such a question, a new approach based on DGGE profiles and BrdU-labeling DNA was carried out. This chapter shows how in the case of a microcosm study it is possible to identify the active oxalotrophic groups over the oxalotrophic community in an OCP system such as the Iroko tree (*Milicia excelsa*), in Cameroon.

Abstract

The oxalate-carbonate pathway (OCP) leads to a potential carbon sink in terrestrial environments. This process is linked to the activity of oxalotrophic bacteria. Although isolation and molecular characterizations are used to study oxalotrophic bacteria, these approaches do not give information on the active oxalotrophs present in soil undergoing the OCP. The aim of this study is to assess the diversity of active oxalotrophic bacteria in soil microcosms using the Bromodeoxyuridine (BrdU) DNA-labeling technique. Soil was collected near an oxalogenic tree (*Milicia excelsa*). Different concentrations of calcium oxalate (0.5, 1, and 4% w/w) were added to the soil microcosms and compared to an untreated control. After 12 days of incubation, a maximal pH of 7.7 was measured for microcosms with oxalate (initial pH 6.4). At this time point, a DGGE profile of the *frc* gene was performed from BrdU-labeled and unlabeled soil DNA. Actinobacteria (*Streptomyces*- and *Kribbella*-like sequences), Gamma- and Beta-Proteobacteria were found as the main active oxalotrophic bacterial groups. This study highlights the relevance of Actinobacteria as members of the active bacterial community and the identification of novel uncultured oxalotrophic groups (i.e. *Kribbella*) active in soils.

a. Introduction

The oxalate-carbonate pathway (OCP) links photosynthetic CO_2 fixation, oxalate synthesis, and calcium carbonate precipitation in tropical soils (Verrecchia, et al., 2006). As long as the calcium source originates from non-carbonate rocks, OCP can lead to a long-term carbon sink as it has been shown for some studied sites in Africa around Iroko trees which are constituting true carbon trapping ecosystems (Cailleau, et al., 2011). Oxalotrophic bacteria have been identified as the key group responsible for the conversion of carbon from oxalate into secondary calcium carbonate. Oxalate catabolism is related to physicochemical changes in the surrounding environment, in particular the shift of soil pH towards alkalinization, which is a key element to recognize the OCP in acidic soils (Braissant, et al., 2002). Previous studies have demonstrated that the shift in pH is due to oxalotrophic activity in Petri dishes (Jayasuriya, 1955, Braissant, et al., 2004), and more recently, the same has been shown in soil microcosms with fungi and bacteria (Martin, et al., 2012).

In the last decades, the study of oxalotrophic bacteria has shifted from culture-based to culture-independent techniques. The design of specific primers for the frc gene to study non-culturable oxalotrophic bacteria (Khammar, et al., 2009) has made possible to assess the diversity and abundance of oxalotrophs in environmental samples. The *frc* gene codes the enzyme formyl-CoA transferase, implicated in the activation of the oxalate molecule to oxalyl-CoA by cycling the CoA moiety from formyl-CoA (Sidhu, et al., 1997). In the oxalotrophs studied so far, oxalate catabolism depends on the action of this enzyme as a first step to yield energy conservation and growth (Dimroth & Schink, 1998, Sahin, 2003).

There are several molecular methods that might allow the analysis of active oxalotrophic bacteria using the frc gene. These methods, which include the recovery and analysis of *frc* mRNA, have been so far unsuccessful due to the difficulties linking tropical field work and RNA stability during transportation of soil samples back to the laboratory. Although RNA fixation has been assayed, this has been so far unsuccessful in our case. Besides, low mRNA yields (below than 0.2 ng μL-1) limit downstream analyses (Bravo et al., unpublished data). An alternative approach is the labeling of DNA with bromodeoxyuridine (BrdU), which allows indirectly to determine active bacteria in specific metabolic processes (Borneman, 1999). The BrdU is a thymidine analog that is assimilated only into DNA from actively replicating cells (Urbach, et al., 1999, Edlund & Jansson, 2008). This means that by separating BrdU-DNA from bulk DNA by immunocapture, one can

analyze a particular functional group that has been labeled and corresponds to a specific metabolism (Hirsch, et al., 2010).

To improve our understanding of the OCP in tropical soils, it is critical to know the bacterial populations that actively carry out the catabolism of calcium oxalate in soils. In addition, the influence of various concentrations of calcium oxalate, a key nutritional factor that might affect the structure of oxalotrophic bacterial communities (Blackmore & Quayle, 1968, Sahin, 2003) is also unknown. Therefore, the aims of this study are to determine the actively replicating part of the community using a BrdU-*frc* gene approach, and to assess the influence of calcium oxalate concentration on oxalotrophic community composition. This is the first time that active oxalotrophic bacteria are identified in a microcosm study as a proxy of the OCP in natural habitats.

b. Material and methods

i. Microcosms design

Microcosms were carried out using soil material collected near a young iroko oxalogenic tree (Milicia excelsa) at the sub-tropical region of Bertoua, Cameroon (4°25' N, 13°36' E). This soil is an Epipetric Calcisol developed in a Ferralsol, following the WRB classification (IUSS, 2006) with an initial pH 6.0. Microcosms with 10 g of soil were prepared in sterile plastic six-well cell culture plates (Cellstar 657 160, Greiner bio-one, Frickenhausen, Germany) with 15 mL of capacity. Various concentrations (0.5%, 1%, and 4 % (w/w)) of monohydrated calcium oxalate (Caox) were amended as the only additional carbon source. Un-amended soil (0%) was kept as a control. Incubation was carried out at 30°C in the dark. The water content was adjusted weekly to 30% of the soil's holding capacity with sterile de-ionized water. All experiments were conducted in six replicates.

ii. Soil pH measurements

A volume of 1.25 mL of de-ionized water was added to 0.525 ± 0.025 g of dried soil (overnight at 105°C), placed on a shaker for 2 h and centrifuged at 16 000 x g for 1 min. The pH_{H2O} was measured in the supernatant with a pH microprobe (Biotrode, Metrohm, Zofingen, Switzerland). A one-way ANOVA test was used to compare pH in the different Caox concentrations.

iii. BrdU labeling

At 0, 5, 10, and 15 days of incubation, three microcosms per Caox concentration were treated with 5-bromo-2'-deoxyuridine, (BrdU B-9285- Sigma Aldrich, München, Germany) in order to label DNA of replicating bacteria. Labeling consisted in the addition of 1 mL of BrdU solution (200 mM) to the entire microcosm (10 g of soil), followed by an additional incubation of 48 h to allow DNA labeling (Hjort, et al., 2007). Three additional microcosms per Caox concentration were amended with 1 mL of sterile de-ionized water before the 48 h incubation.

iv. DNA extraction

BrdU labeled and unlabeled DNA was extracted using the PowerSoil® DNA Isolation Kit (MO BIO Laboratories, Inc, CA, USA). The extractions were done according to the manufacturer's instructions from 1 g of soil, except that DNA was eluted with 30 μL of elution buffer. DNA was quantified using a Nanodrop® spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, USA) and kept at -20°C. DNA concentrations ranged from 6 to 165 ng μL^{-1}.

v. Immunocapture

DNA with incorporated BrdU was purified by immunocapture using antibodies against BrdU (Urbach, et al., 1999, Artursson & Jansson, 2003, Edlund & Jansson, 2006, Edlund & Jansson, 2008). Briefly, monoclonal anti-BrdU antibodies (Sigma Aldrich, St Louis, USA) were mixed at a 1:9 ratio with sheared and denatured herring sperm DNA (Promega, Madison, Wisconsin, USA) and incubated for 1 h at room temperature. Magnetic beads (Dynabeads) coated with goat anti-mouse immunoglobulin G (DYNAL, Oslo, Norway) were washed three times with 1 mg mL^{-1} acetylated bovine serum albumin (BSA) in phosphate-buffered saline (PBS) buffer using a magnetic particle concentrator. Extracted DNA was denatured (heated for 5 min at 100°C and transfer into ice) mixed the herring sperm DNA antibody mixture and incubated for 1 h in the dark at room temperature with agitation. The samples were mixed with the Dynabeads and the incubation was continued for an additional 1 h. After incubation, the samples were washed in 0.5 mL PBS–BSA and the elution of the BrdU-containing DNA fraction was performed by adding 1.7 mM BrdU (in PBS–BSA), and incubated for 1 h in the dark at room temperature.

vi. Denaturing gradient gel electrophoresis (DGGE) profile

The community structure of oxalotrophic bacteria was studied using denaturing gradient gel electrophoresis (DGGE). BrdU-labeled and unlabeled DNA was used as a template for the

amplification of the *frc* gene. A fragment of 155 bp of the *frc* gene was amplified with the primers frc171-f and frc306-r (Khammar, et al., 2009). For DGGE a 40-bp-long GC-clamp (Muyzer, et al., 1993) was attached to the 5' end of the reverse primer. PCR amplification was performed in a final volume of 50 μL. The PCR mix contained: 1X Standard Buffer with 2 mM $MgSO_4$, 0.2 mM dNTPs, 1.25 μM of each primer and 1 U of Taq DNA polymerase (NewEngland, Biolabs, Ipswich, MA, USA). Two μL of DNA (1.6 - 2 ng μL^{-1}) was added as a template. The first denaturation step was performed at 94°C for 5 min, followed by 35 amplification cycles. Cycles consisted of denaturation at 94°C for 30 s, primer annealing at 56°C for 1 min 30 s, and extension at 68°C for 45 s, with a final extension at 68°C for 10 min. The PCR was performed in a thermocycler Bio-Rad MJ Mini PTC-1148. DGGE was performed using a DCode system (BioRad, A.G. München, Germany). The purified PCR products (500 ng in 15 μL) were loaded directly onto the gel with 5 μL of loading buffer 1X (60% sucrose, 0.25% Bromophenol blue, and 1% Xylencyanol). Separation was carried out in 7.5% polyacrylamide gels with a gradient of 40 to 60% of denaturants (100% denaturant solution with 420 g L^{-1} urea and 400 mL L^{-1} deionized formamide in 0,5X TAE). Gels were run during 5 h at 150 V at 60°C. The gel was stained with 0.01% SYBRGold (BioTium Inc. CA, USA) at 4°C in the dark for 30 min. Image was acquired with a Multi-Analyst system (VWR, Fontenay-sous-Bois, France). The normalization, clustering, and band selection were carried out with the software GelCompare II (Sint-Martens-Latem, Belgium) version 4.0.

vii. Identification of active oxalotrophic bacteria

PCR products from selected bands were excised from the DGGE gel and used as template for a PCR with the same conditions described before, except that the primers were used without the GC-clamp. Amplicons were purified and sent for Sanger sequencing at GATC-Biotech AG (Konstanz, Germany). Sequences (121 bp) from 44 bands corresponding to 12 d were used for similarity search against known frc gene sequences by tBLASTx (Altschul, et al., 1997) with the non-redundant nucleotide database at the National Center for Biotechnology Information (NCBI).

c. Results

i. Shift in soil pH

The aim of this microcosm experiment was to assess the dynamics of total and active oxalotrophic bacteria during calcium oxalate (Caox) catabolism in soil. The microcosms were

amended with different concentrations of Caox in order to verify the effect of the substrate concentration. The un-amended microcosm (control) did not show a significant variation in pH (initial pH 6.4 versus final pH 6.3) after 17 days of incubation (Figure 1). In contrast, for each concentration of Caox tested (0.5, 1, and 4%), changes in pH in the microcosms were observed from day 7 and were consistent between biological replicates. The amendments with Caox led to an increase of 1.2 pH units with 1 and 4% of Caox, and almost 1 pH unit with 0.5% of Caox. A one-way ANOVA test showed no significant variation in the final pH for the treatments with different concentrations of Caox (p-value=0.06).

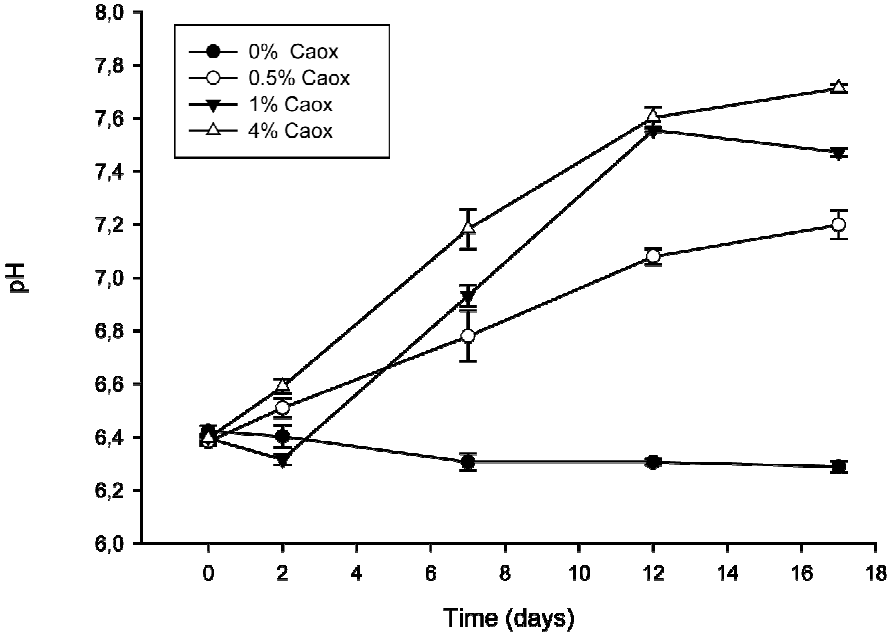

Figure 1. Evolution of soil pH in the amended and control soil microcosms. The amendments consisted in the addition of 0.5%, 1%, and 4% of monohydrate calcium oxalate (Caox). The control is presented as 0% of calcium oxalate. The measurements were conducted in triplicates per each time point. Standard errors are included for each measurement.

ii. Comparison of the BrdU-labeled and unlabeled oxalotrophic bacterial community

Since the evolution of pH could be related with changes in oxalotrophic community structure, a frc-DGGE was carried out in BrdU-labeled (active) and un-labeled (total) DNA from the soil microcosm experiments at 12 days of incubation (Figure 2A), which corresponded to the time with the maximal increase of activity. The frc DGGE profile showed significant differences

between labeled (lanes A) and unlabeled (lanes B) oxalotrophic communities. Minor differences were observed between communities from soil amended with different concentrations of Caox.

Forty-four bands were excised and used as templates for a PCR to identify the *frc* amplicons. For clarity, the bands selected are shown in a scheme aside the DGGE profiles (Figure 2B). Since the same or closely related bacterial species have similar electrophoretic mobilities (i.e. a rate of movement of separating molecules through the polyacrylamide gel; Muyzer & Smalla, 1998, Zhang & Fang, 2000), bands with the same migration rate were classified as mobility species. Twenty mobility species (represented by capital letters in Figure 2B) were identified.

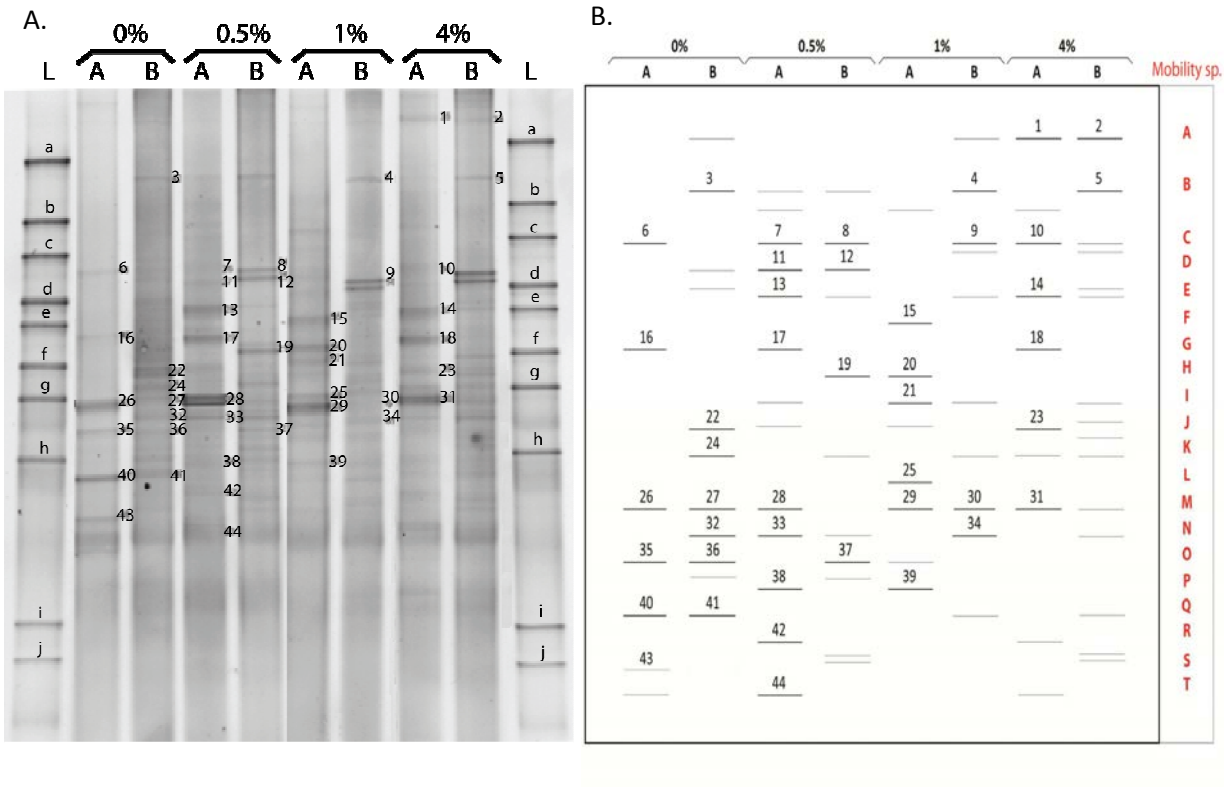

Figure 2 A. DGGE analysis of PCR products for the *frc* gene in soil microcosms amended with different concentrations of Caox at day 12 of incubation. The Caox concentrations are shown on top. Control = 0% of Caox. To differentiate between the total and active fractions, a BrdU labeling was conducted. A = BrdU–treated DNA. B = unlabeled DNA. The ladder (L) consists of *frc* sequences from *Ancylobacter polymorphus* NEU 1210 (a), *Variovorax paradoxus* NEU 2132 (b), *Azospirillum brasilense* NEU 1208 (c), *Methylobacterium extorquens* NEU 44 (d), *Oxalicibacterium flavum* NEU 98 (e), *Cupriavidus necator* NEU 2116 (f), *Pandoraea* sp. NEU 45 (g), *Cupriavidus oxalaticus* NEU 1047 (h), *Streptomyces violaceoruber* NEU 1225 (i), and *Streptomyces flavogriseus* B17DB (j). The numbers correspond to bands excised and sequenced. **B.** Schematic representation of DGGE excised bands. The bands were grouped into electrophoretic mobility species (Mobility sp.) shown on the right.

iii. Identification of active oxalotrophic bacteria

For the identification of the excised bands, tBLASTx search was selected in order to consider the translated product of the frc gene. Results of the tBLASTx are presented in Table 1. Despite the fact that the fragment used for identification is short (155 bp), the percentage of identity ranged from 83 to 99%, confirming that the products corresponded to the *frc* gene. In all the cases when different bands from the same mobility species were considered, an equal or closely related tBLASTx hit was obtained (e.g. for mobility sp. C, the closest relative corresponded to *Streptomyces coelicolor* and *Streptomyces hygroscopicus*). The closest identified relatives corresponded to genera from Alpha- (*Azospirillum*, *Methylobacterium*, *Xanthobacter*, Bradyrhizobium, and Starkeya - like phylotypes), Beta- (*Burkholderia* and *Janthinobacterium* - like phylotypes) proteobacteria, and actinobacteria (*Streptomyces* and *Kribbella* - like phylotypes). Although the majority of the identified groups were known oxalotrophic bacteria, among the Actinobacterial group, several sequences had as first tBLASTx hit *Kribbella flavida* (percentage of identity 94-99%), which is a species not previously known as oxalotrophic.

Table 1. Results of tBLASTx obtained after sequencing of the gene *frc* - small fragment (155 bps). In bold sequences from bands excised in BrdU-labeled DNA are shown. For the electrophoretic position of mobility species (Mobility sp.) see Figure 2b.

DGGE Band No.	Mobility sp.	Identified hit	Accession No.	Id% tBLASTx	e-value	Phylogenetic affiliation
1	**A**	***Methylobacterium radiotolerans***	**CP000316.1**	**95**	**6.E-12**	*Methylobacterium radiotolerans*
2		*Methylobacterium radiotolerans*	CP000316.1	99	9.E-05	
3	**B**	*Bradyrhizobium japonicum*	NC_004463.1	86	5.E-05	*Bradyrhizobium japonicum*
4		*Bradyrhizobium japonicum*	NC_004463.1	88	4.E-10	
5		*Bradyrhizobium japonicum*	NC_004463.1	84	3.E-09	
6	**C**	***Streptomyces coelicolor***	**AL939128.1**	**99**	**1.E-12**	*Streptomyces* sp.
7		***Streptomyces coelicolor***	**AL939128.1**	**96**	**3.E-16**	
8		*Streptomyces hygroscopicus*	CP003275.1	92	7.E-04	
9		*Streptomyces hygroscopicus*	CP003275.1	94		
10		***Streptomyces coelicolor***	**AL939128.1**	**98**	**9.E-09**	
11	**D**	***Methylobacterium extorquens***	**CP000316.1**	**95**	**7.E-05**	*Methylobacterium extorquens*
12		*Methylobacterium extorquens*	FP103042.2	98	2.E-03	
13	**E**	***Streptomyces hygroscopicus***	**CP003275.1**	**99**	**2.E-09**	*Streptomyces* sp.
14		***Streptomyces violaceusniger***	**CP002994.1**	**98**	**2.E-09**	
15	**F**	***Streptomyces avermitilis***	**BA000030.3**	**99**	**3.E-08**	*Streptomyces avermitilis*
16	**G**	***Xanthobacter autotrophicus***	**CP000781.1**	**85**	**1.E-03**	*Xanthobacter autotrophicus*
17		***Xanthobacter autotrophicus***	**CP000781.1**	**88**	**1.E-06**	
18		***Xanthobacter autotrophicus***	**CP000781.1**	**83**	**1.E-05**	
19	**H**	*Streptomyces hygroscopicus*	CP002993.1	86	4.E-11	*Streptomyces hygroscopicus*
20		***Streptomyces hygroscopicus***	**CP003275.1**	**99**	**5.E-13**	
21	**I**	***Streptomyces bingchenggensis***	**CP002047.1**	**99**	**2.E-08**	*Streptomyces bingchenggensis*
22	**J**	*Azospirillum brasilense*	HE577331.1	89	6.E-06	*Azospirillum*

23		***Azospirillum brasilense***	**HE577331.1**	**87**	**3.E-07**	*brasilense*
24	**K**	*Starkeya novella*	CP002026.1	82	1.E+07	*Starkeya novella*
25	**L**	***Streptomyces scabiei***	**FN554889.1**	**99**	**4.E-09**	*Streptomyces scabiei*
26	**M**	***Kribbella flavida***	**CP001736.1**	**97**	**1.E-12**	*Kribbella flavida*
27		*Kribbella flavida*	CP001736.1	99	9.E-11	
28		***Kribbella flavida***	**CP001736.1**	**99**	**1.E-11**	
29		***Kribbella flavida***	**CP001736.1**	**94**	**1.E-12**	
30		*Kribbella flavida*	CP001736.1	99	1.E-10	
31		***Kribbella flavida***	**CP001736.1**	**98**	**3.E-13**	
32	**N**	*Streptomyces davawensis*	HE971709.1	98	1.E-10	*Streptomyces davawensis*
33		***Streptomyces davawensis***	**HE971709.1**	**97**	**2.E-08**	
34		*Streptomyces davawensis*	HE971709.1	97	2.E-09	
35	**O**	***Burkholderia xenovorans***	**CP000271.1**	**77**	**8.E-05**	*Burkholderia xenovorans*
36		*Burkholderia xenovorans*	CP000271.1	96	3.E-09	
37		*Burkholderia xenovorans*	CP000271.1	96	5.E-09	
38	**P**	***Methylobacterium radiotolerans***	**CP000316.1**	**99**	**9.E-05**	*Methylobacterium* sp.
39		***Methylobacterium populi***	**CP001029.1**	**93**	**2.E-04**	
40	**Q**	***Streptomyces violaceusniger***	**CP002994.1**	**91**	**4.E-07**	*Streptomyces violaceusniger*
41		*Streptomyces violaceusniger*	CP002994.1	89	6.E-06	
42	**R**	***Streptomyces cattleya***	**NC_017586.1**	**99**	**5.E-08**	*Streptomyces cattleya*
43	**S**	*Janthinobacterium* sp.	CP000269.1	93	1.E-10	*Janthinobacterium* sp.
44	**T**	***Janthinobacterium* sp.**	**CP000269.1**	**92**	**2.E-08**	*Janthinobacterium* sp.

In order to distinguish the total and active oxalotrophic bacterial communities, the mobility species were sorted based on the presence of the bands in the different treatments. Several groups were identified (Table 2). The first group represented by mobility species A and K, was present in most unlabeled DNA samples, but was only observed at the highest Caox concentration in labeled DNA. The second (mobility species B and N) is present in all unlabeled DNA samples, but only at 0.5% Caox in labeled DNA. The detection of the third group (Mobility species C, E, Q, I, O, D, and H) is more random regarding Caox concentrations, but appears in labeled and unlabeled DNA. The mobility species J appear at 0% in unlabeled DNA and then always in labeled DNA from Caox amended microcosms. The mobility species M appears in all Caox concentrations in labeled DNA and in most unlabeled samples. The sixth group includes mobility species only found in labeled DNA (mobility species T, G, R, P, F, and L) at different Caox concentrations. It is worth mentioning that three out these six mobility species were closely related to Streptomyces. Finally mobility species S was detected in labeled DNA but only at 0% Caox, where soil pH did not change.

Table 2. Classification of mobility species according to the detection in labeled and unlabeled DNA

		Labeled DNA [Caox]%				**Unlabeled DNA [Caox]%**			
Mobility sp.	**Phylogenetic affiliation**	**0**	**0.5**	**1**	**4**	**0**	**0.5**	**1**	**4**
A	*Methylobacterium* - like				■	■		■	■
K	*Starkeya novella* - like				■	■	■	■	■
B	*Bradyrhizobium japonicum* - like		■			■	■	■	■
N	*Streptomyces* - like		■			■	■	■	■
C	*Streptomyces* - like	■	■		■		■	■	■
E	*Streptomyces* - like		■		■		■	■	■
Q	*Streptomyces* - like	■				■		■	■
I	*Streptomyces* - like		■	■				■	■
O	*Burkholderia* - like	■		■		■	■		
D	*Methylobacterium* - like		■			■	■		
H	*Streptomyces* - like			■			■		
J	*Azospirillum* - like		■	■	■	■			
M	*Kribbella flavida* - like	■	■	■	■	■		■	■
T	*Janthinobacterium* - like	■	■		■				
G	*Xanthobacter* - like	■	■		■				
R	*Streptomyces* - like		■		■				
P	*Methylobacterium* - like		■	■					
F	*Streptomyces* - like			■					
L	*Streptomyces* - like			■					
S	*Janthinobacterium* - like	■							

d. Discussion

i. Role of Caox on soil pH and the composition of oxalotrophic communities

Concentrations of Caox observed in different soils in which OCP has been characterized range from 0.015 to up to 0.175 mg g^{-1} of soil (Martin, et al., 2012). More recently, we have observed that values in litter can be up to 1.3 mg g^{-1} of soil (unpublished results). The maximum concentration of Caox used in the present and previous microcosm studies (Martin, et al., 2012) is largely above these values (4 mg g^{-1} of soil). This Caox concentration is the same used for the isolation and culturing of oxalotrophic bacteria (Tamer & Aragno, 1980, Braissant, et al., 2002), and therefore is expected not to be toxic for bacterial growth. The present study shows that the addition of even concentrations as low as 0.5% of Caox stimulates oxalotrophic activity, leading to a shift in the local soil pH (7.2). The increase on pH from 6.4 to 7.2 or 7.7 (for 1 and 4 % of Caox, respectively) demonstrates experimentally for the first time that the input of Caox is one of the limiting factors for bacterial oxalotrophic activity in soil.

Surprisingly, the comparison of different concentrations of Caox showed that a change in the concentration of the amended carbon source is not a driving force that modifies drastically the composition of the oxalotrophic community. Previous studies have demonstrated the selection of similar microbial communities by structurally similar carbon sources, which are metabolized by related biochemical pathways (Wawrik, et al., 2005). Nonetheless, the same study has shown that, if a soil community is enriched on more than one carbon source, changes in the composition of the enriched community are observed. Indeed, several carbon sources have been shown to modify drastically the composition of active metabolically soil bacteria (Monard, et al., 2008). Although other natural carbon sources can be expected in the microcosm experiment, in the case of oxalotrophic bacteria, only Caox amendment appears to be significant.

ii. Identification of active oxalotrophic bacteria

Although the incorporation of BrdU is reported not to be equally effective in all bacteria (Borneman, 1999) and thus some groups can be underestimated, in this study we highlight the use of the BrdU assay to identify a diverse assemblage of active oxalotrophs in microcosm experiments. The 20 mobility species identified were affiliated to bacteria related to Actinobacteria and the divisions Alpha- and Beta- of the class Proteobacteria. Certain genera like *Methylobacterium*, *Xanthobacter*, *Bradyrhizobium*, *Burkholderia*, *Azospirillum*, and *Janthinobacter* have been previously identified as oxalotrophic using culture methods (Sahin, et al., 2008). Some specific species such as *Methylobacterium extorquens* are model bacteria with high metabolic rate when grown on potassium oxalate (consumption rate of 0.32 $\mu M\ h^{-1}$;(Bravo, et al., 2011). Nonetheless, this is the first time that their active metabolic contribution to oxalate catabolism in soil has been demonstrated. Moreover, this is the first time that groups such as *Kribbella* and *Starkeya* are shown as active oxalotrophic bacteria. These bacteria are probably unable to grow in vitro, since they have never been reported in studies dealing with the diversity of cultured oxalotrophic bacteria (Sahin, 2003).

Nonetheless, the limitations of the BrdU method need to be considered in the analysis of the results. For example, groups such as K, B, N that appear at all the concentrations in unlabeled DNA, only appear in one concentration in the labeled DNA. Likewise, the absence of mobility species A, K, B, N, D, and J in BrdU labeled DNA, and the presence of the same mobility species in unlabeled DNA, is a clear evidence of a bias in the BrdU-*frc* detection at 0% of Caox. The opposite was observed with the presence of mobility species C, T, G, and S in labeled DNA and their absence in the unlabeled DNA in the absence of Caox. Further experiments using a

quantitative approach for the detection of specific frc mobility species could help to improve the resolution of these results, as well as to elucidate the detection limit of oxalotrophic mobility species. Thus, the idea that certain populations remain at low intensity or are poorly labeled when no stimulation of Caox is carried out in the system (0%) should be taken into account.

Another issue that needs to be considered in the repeatability of the results. We conducted a rigorous sampling program and considered biological replicates in our analysis. Those replicates were consistent in terms of pH evolution, as well as with our previous results for African soils (Martin, et al., 2012). Nevertheless, the microcosms approach is still a method to try to model activity *in situ* but does not necessarily reproduce it entirely (Bowling, et al., 1980, Fraser & Keddy, 1997, Fraser, 1999). Therefore, it would be important to validate the results obtained, first in other microcosms with other OCP soils, and more importantly, in the field. This is still technically challenging but should be targeted as a priority for future experiments.

The role and metabolic capability of Actinobacteria such as *Streptomyces* and *Kribbella* as oxalotrophs is worth discussing in more details. It has been demonstrated that a *Streptomyces* sp. (strain BV1M3), isolated from a tropical soil, has also a large activity when grown on Kox as sole carbon source (consumption rate of 0.26 $\mu M\ h^{-1}$; (Bravo, et al., 2011). Many studies on the role of Actinobacteria as oxalotrophic bacteria have been primarily concerned with the enumeration and taxonomy of *Streptomyces* (Lechevalier & Lechevalier, 1970, Sahin, 2003, Sahin, 2004). This group is known to be saprophyte (Goodfellow & Williams, 1983). The filamentous morphology and spore dispersion by rain (Gobat, et al., 2004) or attached to arthropods (Ensign, 1978), make *Streptomyces* (and Actinobacteria in general) ideal microorganisms to exploit habitat heterogeneity influenced by the availability of any given substrate (Kassen, 2002), and this could be particularly true for Caox due to its low solubility (Cromack, et al., 1977), probably explaining the importance of *Streptomyces* as active oxalotrophs in the soil microcosms.

Although DGGE (Muyzer & Smalla, 1998) is a technique at the basis of the development of microbial ecology, it is increasingly being displaced by high throughout sequencing approaches. However, for exploratory experiments in which the amount of sampling and the correct timing to observe a meaningful effect (i.e. maximum Caox oxidation) are unknown variables, DGGE is a pertinent compromise of analytical investment. It is clear that the amount of time devoted to obtain a limited number of sequences after band excision is not comparable to the massive amount of data that can be obtained by novel sequencing approaches. Nonetheless, the data generated here constitute a suitable basis for future more targeted experiments, in which

obtaining a comprehensive view of the oxalotrophic community would be justifiable. Finally, attention needs to be paid to the fact that the fragment used in this study is very short and that, ideally for a phylogenetic reconstruction of the community, a more complete amplicon of the *frc* gene would be a better approach. We expect that this will be possible by an increase in our knowledge of bacterial oxalotrophy in a near future, contributing to take full advantage of the potential of new screening and characterizing techniques.

e. Conclusion

This study identifies active oxalotrophic bacterial populations that could be important for the OCP in soil. The results also demonstrated that this molecular method is a powerful tool for screening of active oxalotrophs, which complements conventional methods in microbiology. The use of the BrdU assay combined with other techniques, like isothermal microcalorimetry, will contribute to the analysis of active oxalotrophic bacteria to increase the understanding of their role in the OCP.

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Chapter 4. Isolation and characterization of oxalotrophic bacteria from tropical soils

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What is the diversity of oxalotrophic bacteria in various tropical soils?

Chapter summary

Our knowledge about oxalotrophic bacteria from tropical habitats was incipient before this study. This chapter shows the diversity of culturable oxalotrophic bacteria isolated from OCP systems in three tropical soils in Bolivia, India, and Cameroon. Moreover, this chapter highlights the characterization of metabolic activity of a selected group of oxalotrophic bacteria from the three sampling sites. The use of one of the approaches developed in this study, the IMC (chapter 2) and the metabolic profiles reveal that the strains *Variovorax soli* C18, *Lysobacter* sp. A8, *Agrobacterium* sp. C23, and *Streptomyces achromogenes* A9 are efficient in the oxidation of oxalate in the context of the OCP at each site sampled. Therefore, the ecological role of these strains in the OCP at each system is discussed at the end this chapter.

Abstract

The oxalate-carbonate pathway (OCP) involves the conversion of atmospheric CO_2 into calcium carbonate in tropical, temperate, and probably also semiarid environments. During this process, oxalotrophic bacteria are instrumental because of their capacity to oxidize calcium oxalate (Caox) from plant or fungal origin. However, the diversity and metabolic plasticity of oxalotrophs as part of this pathway is unknown. The aim of this study is the characterization of oxalotrophic bacteria in tropical OCP systems in Bolivia (tree *Terminalia oblonga*), India (tree *Terminalia bellirica*), and Cameroon (tree *Milicia excelsa*). Ninety-five isolates were obtained by plating in a selective medium with Caox. Twenty-three genera were identified by sequencing of the 16S rRNA gene, six of which had not been previously reported as oxalotrophs (*Ensifer*, *Lysobacter*, *Afipia*, *Sphingomonas*, *Polaromonas*, and *Terrabacter*). Ten strains were selected based on their Caox dissolution capacity. Kinetic curves in batch cultures and microcalorimetry analyses showed that *Variovorax soli* C18, *Lysobacter* sp. A8, and *Agrobacterium* sp. C23 have the highest oxalate consumption rates with values of 0.240, 0.221, and 0.209 μM h^{-1}, respectively. *Streptomyces achromogenes* A9 displayed the largest metabolic plasticity, consuming 18 different carbon sources besides Caox. The ecology of the strains is discussed in the context of the OCP.

a. Introduction.

The atmospheric concentrations of CO_2 have increased from 270 to 350 ppm in the last 150 years (Archer, et al., 1995). This tendency has accelerated during the past 18 years with an increase from 350 ppm to 397 ppm (Tans, 2013). Moreover, it is known that there is a positive feedback between global climate and carbon cycling (Friedlingstein, et al., 2001). Although in tropical regions, the majority of the models predict an increase in the photosynthetic capacity of plants (Albert, et al., 2011), higher temperatures leading to increased evaporation rates would induce soil drought and the dieback of the rainforest in the Amazon (Luo, 2007), subtropical India (Lal, 2004), and subtropical areas from Africa (Cao, et al., 2001). This means that, while global and local drivers will accelerate CO_2 emissions, the tropical forest will decrease its dynamic capacity to regulate the carbon cycle around the world (Raupach, et al., 2007). Several strategies have been suggested to tackle the effect of elevated CO_2 concentrations in the atmosphere. Within the repertoire of geobiological solutions, the terrestrial oxalate-carbonate pathway (OCP) seems to be relevant in several ways. It has been demonstrated that the OCP can constitute a carbon sink through the storage of carbon in the form of secondary carbonate in tropical soils (Cailleau, et al., 2004).

The OCP is a biogeochemical pathway consisting of an open system activated by plants, fungi, and bacteria, in which redox reactions of calcium oxalate (Caox) occur thanks to the metabolic activity of a functional group of so-called oxalotrophic bacteria (Verrecchia, et al., 2006). The presence and activity of oxalotrophic bacteria have been shown to contribute with $CaCO_3$ precipitation and thus CO_2 storage (Braissant, et al., 2004). However, little is known about the diversity of oxalotrophic bacteria involved in the OCP. Some genera like *Methylobacterium*, *Pseudomonas*, *Xanthobacter*, *Azospirillum*, and *Burkholderia* have been identified as oxalotrophs (Tamer, et al., 2002). So far, 54 phylogenetically non-related species, in particular *Azospirillum* (Sahin, 2004) and *Methylobacterium* (Sahin, et al., 2008), have been reported as oxalotrophs in terrestrial environments (Sahin, 2003).

In subtropical soils, only a few studies have been performed to understand the ecological role of bacteria in the OCP using the oxalogenic tree *Milicia excelsa* as a model (Braissant, et al., 2004). However, a study on the oxalotrophic diversity in different soils influenced by the OCP, as well as the characterization of oxalotrophic bacteria from tropical soils, is missing. Therefore, the aim of this study was to provide information on the missing identity and physiology of the key players

in the OCP. First, we assessed the diversity of culturable oxalotrophic bacteria in three tropical OCP systems in Bolivia, India, and Cameroon. Secondly, we characterized the metabolic plasticity of oxalotrophic bacteria selected from a pool of environmental strains. To accomplish the aims, several methods were used including enrichment and isolation, and amplification and partial sequencing of 16S rRNA gene (Muyzer, et al., 1993). In addition, analytical tools, which have been developed or modified for oxalotrophic studies, were used for a metabolic characterization of a subset of the environmental strains. Such methods included the measurement of metabolic activity by isothermal microcalorimetry (Bravo, et al., 2011) and characterization of metabolic plasticity by BIOLOG assays (Garland & Mills, 1991).

b. Materials and Methods

i. Origin of soil samples

The occurrence of oxalotrophic bacteria in soils was studied in regions of the world in which the OCP was recognized. The soil samples were collected from Bolivia, India, and Cameroon (i.e. three different continents of the tropical belt). Detailed geographical information is shown in Table 1. At each sampling site, two soil profiles were prepared and used for microbiological studies. The first profile or profile A (up to 170 cm deep) was located near (15 - 50 cm) the trunk of an oxalogenic tree. The second profile (up to 150 cm deep), designated profile C, was carried out at 10 to 14 m from the studied tree and corresponded to the control soil assumed to be unaffected by the Caox flux from the tree. Hydrochloric acid test was performed on soil grains and tree tissues (Cailleau, et al., 2004). This test demonstrated the presence of carbonate. A local increase of soil pH in profile A, and the presence of carbonate either in the soil or in the tree tissues, indicated the activity of the OCP in the field.

Table 1. Origin and geographical characteristics of soil samples.

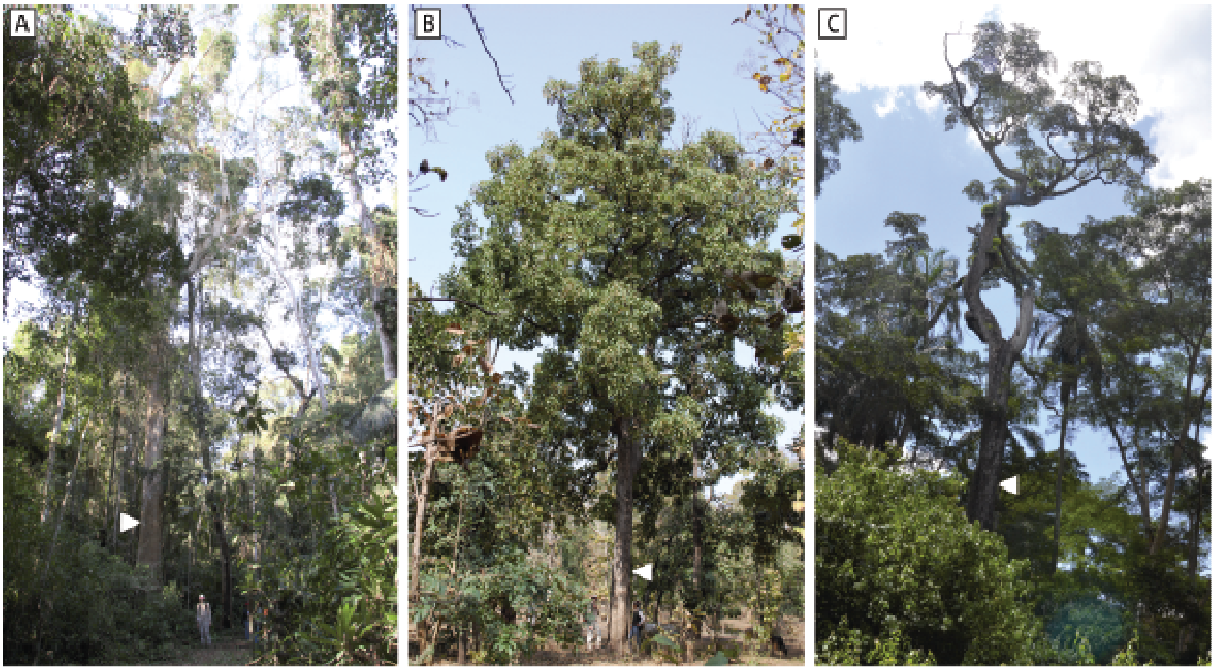
Country	Climate	Average T° [°C]	Soil type	Oxalogenic plant	Geographical Position [Coordinates]
Bolivia	Warm and humide tropical	25	Sandy loam, sandy clay; color brown to very dark brown-gray	*Terminalia oblonga* (Exell.) – Verdolago amarillo	Valle de Inicua (15°23' S, 67° 20' W)
India	Humide sub-tropical	23	Granular - Boudelkhand, color brown to yellow-red	*Terminalia bellirica* (Gaertn.) - Bahera	Panna Tiger Reserve (24°43'N 80°00'E)
Cameroon	Subtropical with four seasons	24	Biogenic loose Calcarisol developed on Ferrallitisol	*Milicia excelsa* – Iroko	Golambela (4°25' N, 13°36' E)

In the site of the Inicua valley, Bolivia (supplementary Fig. 1a), a thorough geological study led to the discovery of outcrops showing sandstones with some carbonate cements, constituting the

substratum on which alluvium laid. Soil profile A (170 cm) was excavated at 15 cm from trunk of *Terminalia oblonga* (Verdolago Amarillo). Profile C, at more than 10 m from the studied tree, was 150 cm deep. The *T. oblonga* specimen presented buttresses on the flank making difficult to measure its diameter. At breast height, the diameter was 170 cm while above the buttresses (3.5 - 4 m from the ground) it was evaluated at 110 cm.

Near to the sampled *Terminalia bellirica* (Bahera) tree in India (supplementary Fig. 1b), the soil parent rock was carbonate-free, the surrounding outcrops being composed of granite and sandstone, the site being located at the edge of a granite intrusion. Soil grains seem to be mostly composed of quartz and feldspars originating from this granite. The topography was flat and the tree selected was one of the largest specimens observed in the explored area. The diameter of the tree was 80 cm and the age was estimated to 50 years (forest officer estimation).

The tree *Milicia excelsa* (Iroko) in Cameroon (Supplementary Fig. 1c) was located in an ancient syntectonic granite area. The diameter of the tree was 2.5 m and the age was estimated between 200 and 250 years. The soil below the hollow trunk contained considerable amounts of carbonate suggesting a very active and efficient OCP.



Supplementary Figure 1. Image of the three trees studied. A= *Terminalia oblonga*, Bolivia; B=*Terminalia bellirica,* India; C= *Milicia excelsa*, Cameroon. White triangles point to the trees.

ii. Isolation of oxalotrophic strains

One gram of soil per sample was used to perform dilutions (from 10^{-1} to 10^{-7}) in 1% saline solution. For culturing, 0.1 mL from the odd dilutions was spread onto solid modified DSM81 medium (Braissant, et al., 2002), containing 4 g L^{-1} of calcium oxalate monohydrate (Caox; Fluka 21201, Sigma Aldrich, Munich, Germany) as carbon source instead of solution C (5% NaHCO3) (Tamer & Aragno, 1980). The modified medium was designated Schlegel AB+Caox. The medium was poured in two solid layers in the Petri dish. The first layer (ca. 20 mL) was prepared from solutions A and B without the addition of the carbon source. The upper layer (ca. 5 mL) was prepared using the same solutions supplemented with Caox. The composition of solution A was (per L of distilled water): $Na_2HPO_4.12H_2O$ 9 g; KH_2PO_4 1.5 g; NH_4Cl 1 g; $MgSO_4.7H_2O$ 0.2 g. One mL of trace-element solution DSM27 was added. The final pH of solution A was adjusted to 7.2. The components of solution B were (in 250 mL of distilled water): $Fe(NH_4)$-citrate 0.125 g and $CaCl_2.H_2O$ 0.250 g. Solid medium was prepared by adding 1.6% agar (Biolife Italiana, Milan, Italy). The ratio of solution A to B per layer was 100:1 (v/v). Both A and B solutions were sterilized in separate flasks due to immobilization of the KH_2PO_4 with $Fe(NH_4)$-citrate. Sterilization was performed at 1 Pa., 120°C, during 20 min. The Petri dishes were incubated at 20±2°C during 4 weeks.

Schlegel AB+Caox medium has been used in oxalotrophic studies as first criteria to select oxalotrophic bacteria. The clear halos around the bacterial colonies are indicative of Caox consumption (Tamer & Aragno, 1980). Therefore, the colonies showing dissolution halos were selected as positive oxalotrophic strains and slanted in fresh medium. The isolates were purified by successive passages. Colony morphology, Gram-staining and cell morphology were obtained by direct observation or under the light microscope (See Supplementary Table 1).

iii. Identification of pure cultures using the 16S rRNA gene

Pure cultures in solid Schlegel AB+Caox were used to perform DNA extractions using the Analytik Jena InnuPrep Bacteria DNA extraction kit (Analytik Jena AG, Jena, Germany), according to the manufacturer's instructions with modifications for Gram-positive bacteria. A sonication pre-step was performed on the biomass using a Branson Sonifier 250 (Branson Ultrasonic Co, Danbury, USA) at 20 KHz and 200 Watts on output voltage at 20% of pulse mode of duty-cycle. The sonication was performed during 7 s in order to dissolve flocks formed by filamentous isolates. Most of the time, this was a necessary step to obtain a good DNA yield. In the digestion step, the incubation time with Lysozyme was increased from 30 to 45 min at

37°C. The incubation with Lysis solution was increased from 10 to 20 min. at 50°C. DNA extracts were quantified using a Nanodrop® spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, USA). DNA concentration ranged from 13 ng µL-1 to 92 ng μL^{-1}. The DNA extracts were conserved at -20°C in 100 µL of the elution buffer provided with the extraction kit.

PCR amplifications of a partial fragment of the 16S rRNA gene were carried out using the primers Eub9_27 forward (5'-GAGTTTGATCCTGGCTCAG-3'), and Eub1542 reverse (5'-AGAAAGGAGGTGATCCAGCC-3'; (Liesack, et al., 1991). The PCR reaction mix contained (final concentration in 50 µL of final volume) 1X Buffer with 2 mM $MgSO_4$ (Standard Buffer Biolabs, New England, Ipswich, MA, USA), 0.2 mM dNTPs mix (Promega AG, Dübendorf, Switzerland), 0.2 µM of each primer, and 1 U of Standard DNA Taq polymerase (Biolabs, New England, Ipswich, MA, USA). A total of 2 µL of diluted DNA (ca. 1.6-2 µg μL^{-1} of DNA) was added as a template for each reaction. The initial denaturation was carried out at 94°C for 4 min 30 s, followed by 30 cycles consisting of denaturation at the same temperature for 30 s, primer annealing at 60°C for 30 s, and extension at 68°C for 1 min 30 s. A final extension was performed at 68°C for 10 min. PCR reactions were carried out in a thermocycler Sensoquest Labcycler (Witec A.G., Göttingen, Germany). PCR products were visualized by gel electrophoresis using 1% agarose gels run 30 min at 90 V and 60 mA in a horizontal electrophoresis chamber (VWR, Fontenay-sous-Bois, France) with 200mL of TBE buffer 0.5 X.

Purification of PCR products was carried out using 96-well filtering plates from Millipore (Millipore AG, Zug, Switzerland). The PCR product was premixed with an equal volume of TE buffer 1 X and filtered during 15 min. Fifty µL of nanopure sterile water were added. The amplicons were quantified using a Nanodrop® spectrophotometer, and sent for Sanger sequencing at GATC-Biotech AG (Konstanz, Germany). DNA concentration from the amplicons sent for sequencing ranged from 24 to 82 ng μL^{-1}. The search for similarity amongst sequences from the gene 16S rRNA was performed using BLASTn (Altschul, et al., 1997) comparing the query sequence with the 16S rRNA gene sequences available in the public nucleotide databases at the National Center for Biotechnology Information (NCBI). The 16S rRNA gene sequences from the isolates have been deposited in GenBank under accession numbers expecting to be assigned (Sequences submitted to NCBI on July 1st 2013).

iv. Kinetic growth using Caox

Batch cultures were carried out for ten selected oxalotrophic strains in order to obtain growth kinetic parameters. The selection of the strains was made based on the Caox dissolution halo

around the purified colonies grown on solid Schlegel AB+Caox (supplementary Table 1). Batch cultures were performed using 20 mL of liquid Angle medium (Angle, et al., 1991) with Caox as carbon source (4 g L^{-1}), incubated in a shaker incubator at 20±2°C and 150 rpm (Lab-shaker, Adolf Kuhner AG, Basel, Switzerland) during 11 days. Biomass was quantified every day using total protein content measured with the Quick StartTM kit (BioRad AG, Munich, Germany) as reported previously (Bradford, 1976), in a Genesys 10S UV-VIS spectrophotometer Thermo Scientific (Fischer Scientific AG, Wohlen, Switzerland) at 600 nm of wavelength. In parallel, samples were collected to evaluate the consumption of oxalate using HPLC. The chromatograms were obtained using a HPLC 110 chromatograph (Agilent Technologies AG, Basel, Switzerland) with a diode array detector (DAD - 210nm UV) and a column for reverse phase RPC18. Caox was extracted according to previous studies for oxalogenic fungi (Schilling & Jellison, 2004), with modifications for oxalotrophic bacteria reported in a previous study (Bravo, et al., 2011). A standard of Caox was prepared to calibrate the oxalate retention time (tR), baseline resolution, and column efficiency. The standard was performed with three replicates consisting of 1 mL of 20 mM H_2SO_4 HPLC-grade and the same concentration of Caox used for kinetic growth curves. Retention time and peak area were used for quantification of Caox.

v. Oxalotrophic metabolism assessed using isothermal microcalorimetry (IMC)

The same strains were characterized by IMC in order to evaluate oxalotrophic activity. *Escherichia coli* K-12 was used as a negative control as this strain has been reported as a non-oxalate oxidizing bacteria (Turroni, et al., 2007). All cultures were maintained in nutrient broth or nutrient agar (NB/NA) media (Difco, Kansas, USA) for regular transfers.

To perform the microcalorimetric assay, all strains with the exception of *E. coli*, were pre-incubated on solid Angle medium supplemented with 4 g L^{-1} of Caox. *E. coli* was pre-inoculated on nutrient agar (NA). Replicate cultures were performed in 4 mL microcalorimetric ampoules filled with 2 mL of slanted solid Angle's medium to which 4 g L^{-1} of potassium oxalate (Kox; pH = 7.0) was added. Inoculation was carried out with an inoculation loop, ensuring that bacteria grow as a loan. The measurements were performed in a 48 channels isothermal heat conduction microcalorimeter (TAM III, Waters/TA Instruments, Delaware, USA). The setup of the experiment was carried out as previously described (Bravo, et al., 2011). Briefly, the temperature of the microcalorimeter thermostat was set for growth of environmental bacteria at 25°C. After stable temperature conditions were obtained, each measuring channel was calibrated using a built-

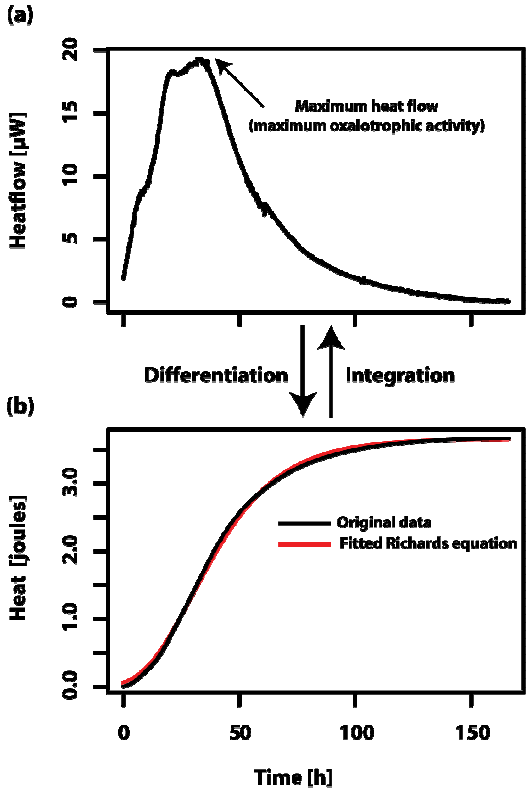
in electrical heater of known power. Microcalorimetric ampoules containing sterile Angle medium were used as blanks.

vi. IMC Data analysis

Analysis of the heat flow (thermograms) was used for calculating kinetic parameters. Considering $1\mu W = 1\mu J \bullet s^{-1}$, the maximum heat flow (Supplementary Fig. 2a) was used to calculate the maximum oxalotrophic activity assuming the following reaction:

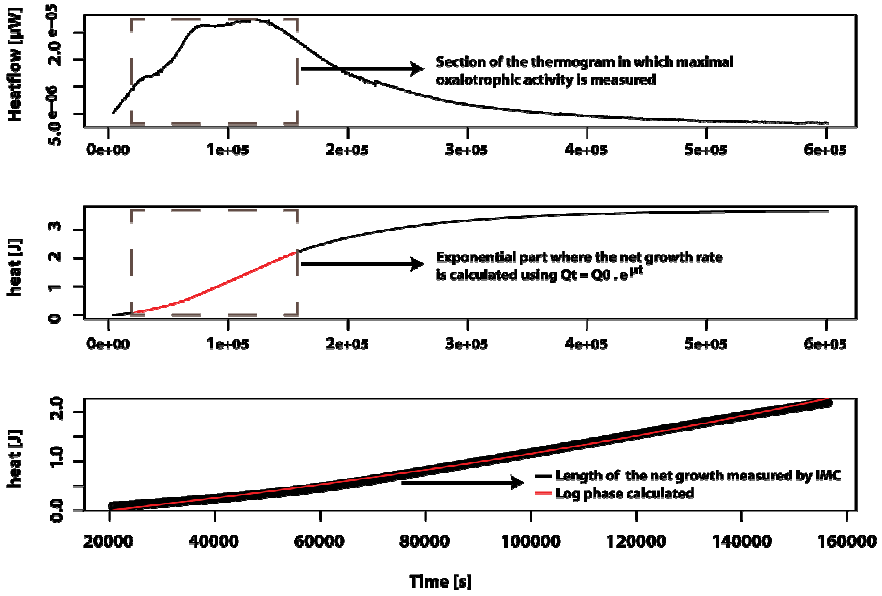
$$2H_2C_2O_4 + O_2 \rightarrow 4CO_2 + 2H_2O$$

with a reaction enthalpy of $\Delta H°'$ of $-499 KJ \bullet mole^{-1}$ (standard enthalpies of formations of reactants and products were obtained elsewhere; (Dean, 1999). This calculation assumes no heat production by biomass formation since biomass yield on oxalate is low.



Supplementary Figure 2. Schematic representation showing the relationship between our calorimetric measurements expressed as heat-flow (**a**), which corresponds to the microbial activity derived from oxalate consumption, and the evolution of heat expressed in Jules (**b**), which is a proxy for products resulting from oxalotrophic activity (i.e. biomass or CO_2). The maximum heat-flow (arrow) is directly proportional to the maximum oxalotrophic activity of the strains selected in this study.

The heat over time curve was obtained by integrating the heat flow data to calculate the net growth rate by fitting an exponential model ($Qt = Q0 \bullet e^{\mu t}$, where Q represents heat) over the exponential part of the curve (See supplementary Fig. 3). Similarly, the maximum growth rate was calculated by fitting the modified Richard's equation (Zwietering, et al., 1990, Braissant, et al., 2013) to the complete heat over time curve (Supplementary Fig. 2b). For every strain, these calculations were performed on three replicates.



Supplementary Figure 3. Sketch explaining how the net growth was calculated using thermograms obtained by isothermal microcalorimetry. The kinetic growth parameter of net growth rate was calculated applying an exponential model fitting heat as a thermodynamic response of bacterial growth.

vii. Metabolic characterization of oxalotrophic strains using BIOLOG™

Ten SF-N2 MicroPlates were inoculated with the selected oxalotrophic strains (0.15±0.02 mL per well). The biomass was recovered from pre-cultures grown in Petri dishes with Schlegel AB+Caox medium and resuspended in 17 mL 1% saline solution. The 96-well BIOLOG SF-N2 MicroPlates system (BIOLOG, Inc. Hayward, CA, USA) comprises 95 substrate-containing wells and a control well without carbon source. It relies on the redox dye tetrazolium violet to detect respiration represented by NADH formation. The substrates consisted of polymers, carbohydrates, esters, carboxylic acids, amides, amino acids, aromatic chemicals amines, alcohols, and phosphorylated chemicals (Garland & Mills, 1991). The microplates were incubated at 20±2°C during a week. The optical density (OD) measurements were carried out at 590 nm using a Microplate Spectrophotometer Subnet 28 (Berthold Technologies GmbH., Regensdorf, Switzerland), with the software DigiRead (ASYS Hitech, Cambridge, UK).

viii. BIOLOG Data analysis

Substrate consumption was considered positive or negative (qualitative characterization) using two complementary criteria. The first one consisted of the difference between final and initial OD values for each well containing a carbon source. This was compared with the difference between final and initial OD of the well without carbon source (accounting for changes in OD due to desiccation during the incubation). A positive consumption of substrate was assumed when the difference in OD for the well with the carbon source was 1.75 times larger than the OD difference for the control well. A second parameter consisted on the color development in each well plate. A picture of the microplate was taken using a camera Canon SX30is Powershot (Canon, Tokyo, Japan) at 10 cm from the plate. The picture was taken at the end of the incubation when the final lecture of microplates was carried out by spectrophotometry. The developed color was compared using the software Image J Macro software (National Institutes of Health, Maryland, USA). The saturation color per pixel and well were calculated. The measurement was expressed as average well color development (AWCD). AWCD was derived from the mean difference between color scale values of the 95 response wells (containing sole carbon sources), R, and the color scale value of the control well (without a carbon source), C. The difference was calculated using the equation $[\Sigma(C - R)]/95$ reported elsewhere (Garland & Mills, 1991). When the AWCD value of the wells with a carbon source was superior to the control, the test was considered as positive.

c. Results

i. Identification of oxalotrophic strains

Ninety-five oxalotrophic strains were isolated and identified in this study. Thirty-two strains were Gram-positive, and 63 were Gram-negative. The most abundant class was Gamma-Proteobacteria with 30 strains, followed by Actinobacteria and Beta-Proteobacteria with 22 and 20 strains, respectively. In a minor proportion were found Alpha-Proteobacteria and Bacilli with 13 and 10 strains, respectively (Fig. 1). The genera *Stenotrophomonas*, *Variovorax*, *Bacillus*, and *Streptomyces* were the most abundant, representing the classes Gamma- and Beta- Proteobacteria, Bacilli, and Actinobacteria. *Stenotrophomonas* spp., the most commonly encountered genus isolated in Bolivian and Cameroon, was not found in India. The strain *Acidovorax* sp. B11 was found only in Bolivian soil samples, while the strains *Ensifer adhaerens* C17, *Rhodospirillum centenum* C34, and *Pseudomonas* sp. C40 were isolated only from Cameroon. The same was observed for the strains

Sphingomonas sp. A34, *Polaromonas jejeuensis* A35, and *Terrabacter* sp. A46, isolated from Indian soil samples.

A distribution of the genera isolated from each tropical OCP system is presented in Fig. 2. From the 23 genera observed, six have not been previously reported as containing oxalotrophic species (*Sphingomonas*, *Afipia*, *Rhodospirillum*, *Polaromonas*, *Terrabacter*, and *Ensifer*). The lowest richness was obtained in Bolivia with seven genera, followed by India (9 genera), and finally Cameroon, which was the richer OCP system with 14 genera. A considerable presence of *Streptomyces* sp. and *Paenibacillus* sp. was observed in soil from India (18 strains over 27), emphasizing that Gram-positive bacteria seem to be selected in this oxalogenic environment.

Twenty-nine strains were isolated and identified from the soil profiles in Bolivia (Fig. 1). Fifteen strains were isolated from profile A (under the influence of the tree) and 14 from profile C (control soil far from the tree). In both cases, the strains were isolated from soil between 1 and 76 cm. The most common genus was *Stenotrophomonas* (14 strains), with *Stenotrophomonas maltophilia* as the closest related species for most of the strains. Nine strains of *Stenotrophomonas* spp. were found in soil from profile C and five in profile A. Eight strains closely related to the genus *Streptomyces* were also isolated. Three strains were related to *Xanthomonas*. Two strains were related to *Variovorax*, and genera such as *Agrobacterium* and *Bacillus* were found in minor proportion (one strain each).

Twenty-seven strains were isolated and identified in soil samples from profile A in India (Fig. 1). The depths at which the strains were isolated corresponded mainly to the 5 upper most cm, while three strains were isolated from 50 to 102 cm. Actinobacteria was the most common group isolated (12 strains), corresponding mostly to strains related to the genus *Streptomyces* and one strain belonging to *Terrabacter* (strain A46). Seven strains belonging to *Paenibacillus* sp. and fourth belonging to *Afipia* sp. were found from 1 to 50 cm. A minor proportion of genera like *Polaromonas*, *Rhizobium*, *Sphingomonas*, and *Bacillus* were also found (one strain each).

Thirty-nine strains were isolated and identified in soil samples from profile A in Cameroon (Fig. 1). The strains were isolated from 1 to 56 cm in the soil profile. The genera *Stenotrophomonas* and *Variovorax* were the most common with eight strains each. These genera were isolated through the profile (1 to 46 cm). Four strains were closely related to *Alcaligenes*. Three strains were closely related to *Achromobacter* and *Agrobacterium*. Two strains belonged to *Lysobacter*, *Cupriavidus*, and *Sinorhizobium*. Other genera such as *Pseudomonas*, *Rhizobium*, *Rhodospirillum*, *Ensifer*, *Paenibacillus*, *Streptomyces*, and *Arthrobacter* were found in minor proportion (one strain each).

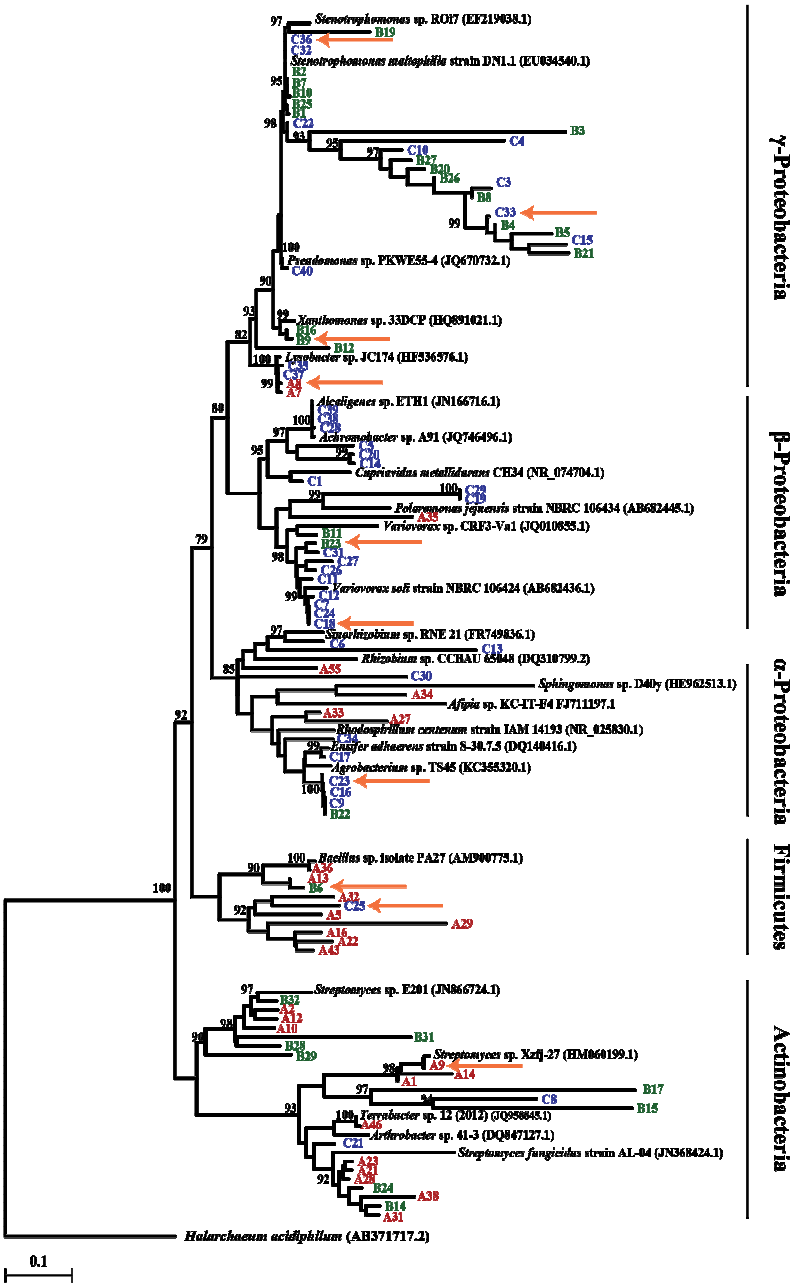
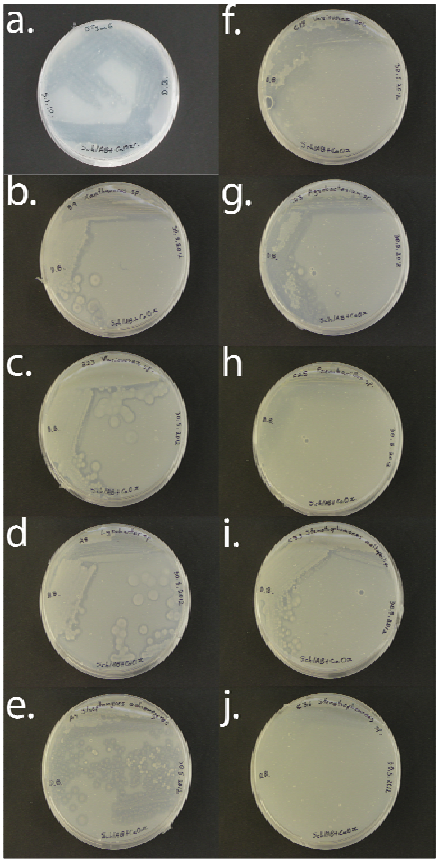


Figure 1. Phylogenetic relationship of oxalotrophic bacteria isolated from Bolivia (B), Cameroon (C) and India (A), based on partial sequences of the 16S rRNA gene. The sequence of *Halarchaeum acidiphilum* (Archaea) was selected as outgroup. Bootstrap values greater than 50% are shown at the nodes. The phylogenetic tree was constructed with the neighbour-joining method, using the TREECON software. The strains selected for further studies are shown with an arrow.

ii. Description of selected oxalotrophic strains

All oxalotrophic bacteria grown in Schlegel AB+Caox presented a degradation halo around the colonies. A comparison of the halos led to the selection of ten oxalotrophic strains for further characterization (Supplementary Table 1). A complete description of the selected strains is presented in table 2. Three strains from Bolivia, two from Indian, and five from Cameroon were included. Four strains belonged to Gamma-Proteobacteria (*Xanthomonas* sp. B9, *Lysobacter* sp. A8, *Stenotrophomonas maltophilia* C33, and *Stenotrophomonas* sp. C36), two to Beta-Proteobacteria (*Variovorax* sp. B23 and *Variovorax soli* C18), two to Bacilli (*Bacillus* sp. B6 and *Paenibacillus* sp. C25), one to Alpha-Proteobacteria (Agrobacterium sp. C23), and one to Actinobacteria (*Streptomyces achromogenes* A9).



Supplementary Figure 4. Strains selected for further characterization. (**a.**) B6 – *Bacillus* sp.; (**b.**) B9–*Xanthomonas* sp.; (**c.**) B23–*Variovorax* sp.; (**d.**) A8–*Lysobacter* sp.; (**e.**) A9-*Streptomyces achromogenes* (**f.**) C18–*Variovorax soli*; (**g.**) C23–*Agrobacterium* sp.; (**h.**) C25–*Paenibacillus* sp.; (**i.**) C33–*Stenotrophomonas maltophilia*; (**j.**) C36–*Stenotrophomonas* sp.

Table 2. Selected oxalotrophic strains. Phylogenetic identification (Class) based on the 16S rRNA gene sequence showing the first BLAST hit in GenBank (Firs BLAST hit) together with the percentage of identity (ID BLAST) with the blasted sequence (Accession N° at the fifth column of this table). Colony description corresponds to the morphology after growth in solid Schlegel AB medium with Caox as sole carbon source. The extent of the clearing Caox halo is indicated (Halo). Gram staining was carried out after 5 d of incubation (Gram). The presence of flagella and motility mode were obtained from literature (Juhnke & des Jardin, 1989, Kamoun & Kado, 1990, Messini & Favilli, 1990, Kim*, et al.*, 2006, Turroni*, et al.*, 2007, Ingham & Jacob, 2008, Ryan*, et al.*, 2009). Soil profile A (positive) corresponds to soil sampled at one side to the oxalogenic tree (*Terminalia oblonga* for Bolivia, *Terminalia bellirica* for India, and *Milicia excelsa* for Cameroon, respectively). Profile C (control) corresponds to soil collected distantly from the tree trunk.

Strain	Class	First BLAST hit	ID BLAST	Accession N°.	Colony description	Halo [cm]	Gram	Flagella	Motility	soil Profile	Depth [cm]
B6	*Bacilli*	*Bacillus* sp.	97	AM900775.1	Brown, small, mucoid	0.5	+	+	Swarming	C	0-10
B9	γ-Proteobacteria	*Xanthomonas* sp.	99	HQ891021.1	Yellow -Brown, irregular border, small, punctiform and powdery	0.6	-	+	Switching	C	0-10
B23	β-Proteobacteria	*Variovorax* sp.	96	JQ010855.1	Brown, irregular border,,diminutes, consistent, small points	0.7	-	+	Swarming	A	0-10
A8	γ-Proteobacteria	*Lysobacter* sp.	99	FR667176.1	Opaque centric, median	0.7	-	-	Gliding	A	0-1
A9	Actinobacteria	*Streptomyces achromogenes*	98	JN400102.1	brown, irregular border, halo dull, double ring, powdery, small	0.8	+	-	Filamentous	A	0-1
C18	β-Proteobacteria	*Variovorax soli*	89	AB682436.1	Cream, translucent, concentric spirals, dull, irregular border	0.8	-	+	Filamentous	A	12-14
C23	α-Proteobacteria	*Agrobacterium* sp.	99	JF730141.1	Gray-Brown, diminutes, powdery	1.3	-	+	Swarming	A	16-17
C25	*Bacilli*	*Paenibacillus* sp.	92	CP001656.1	Cream, translucent, irregular border, median	0.5	+	+	Swarming	A	30-32
C33	γ-Proteobacteria	*Stenotrophomonas maltophilia*	96	HQ434490.1	Brown, powdery, small, dull	0.7	-	-	Gliding	A	45-46
C36	γ-Proteobacteria	*Stenotrophomonas* sp.	99	EF219038.1	Gray, powdery, punctiform, small, irregular border	0.6	-	-	Gliding	A	45-46

iii. Growth curves

The growth kinetics of the selected oxalotrophic strains in batch cultures using liquid Schlegel AB+Caox medium are presented in Figure 3. The growth of the strains was measured during 11 days of incubation. For all the strains from Bolivia and India (Fig. 3a), and the strain C25 (Cameroon, Fig. 3b), growth was observed during the first six days of incubation, followed by a phase of decline. In the case of the two Bacilli (B6 and C25) the maximum growth was observed after five days. Growth for the other four strains from Cameroon was recorded up to eight days before the decline phase.

The strain *S. achromogenes* A9 isolated from India showed the highest biomass production with 8 μg mL^{-1} of protein measured at six days (Fig. 3a). *V. soli* C18, *Agrobacterium* sp. C23, and *Stenotrophomonas* sp. C36, all isolated from Cameroon, showed a maximum biomass production of 5.4 μg mL^{-1} each (Fig. 3b). In contrast, the strains *S. maltophilia* C33, *Paenibacillus* sp. C25, and *Bacillus* sp. B6 showed a very sparse growth with a maximum biomass production of 3.3, 2.2, and 1.9 μg mL^{-1}, respectively. Oxalate consumption parallel to growth was shown using HPLC. The

initial concentration of Caox was 2.500 μg mL^{-1} in batch cultures. Nonetheless, the dissolved concentration of Caox after acid extraction ranged from 1.100 to 1.900 μg mL^{-1} for Bolivian and Indian strains (Fig. 3c), and between 1.100 to 1.500 μg mL^{-1} for Cameroon (Fig. d). For all the cultures, oxalate was consumed steadily during growth, regardless of the biomass produced, and even during the decline phase.

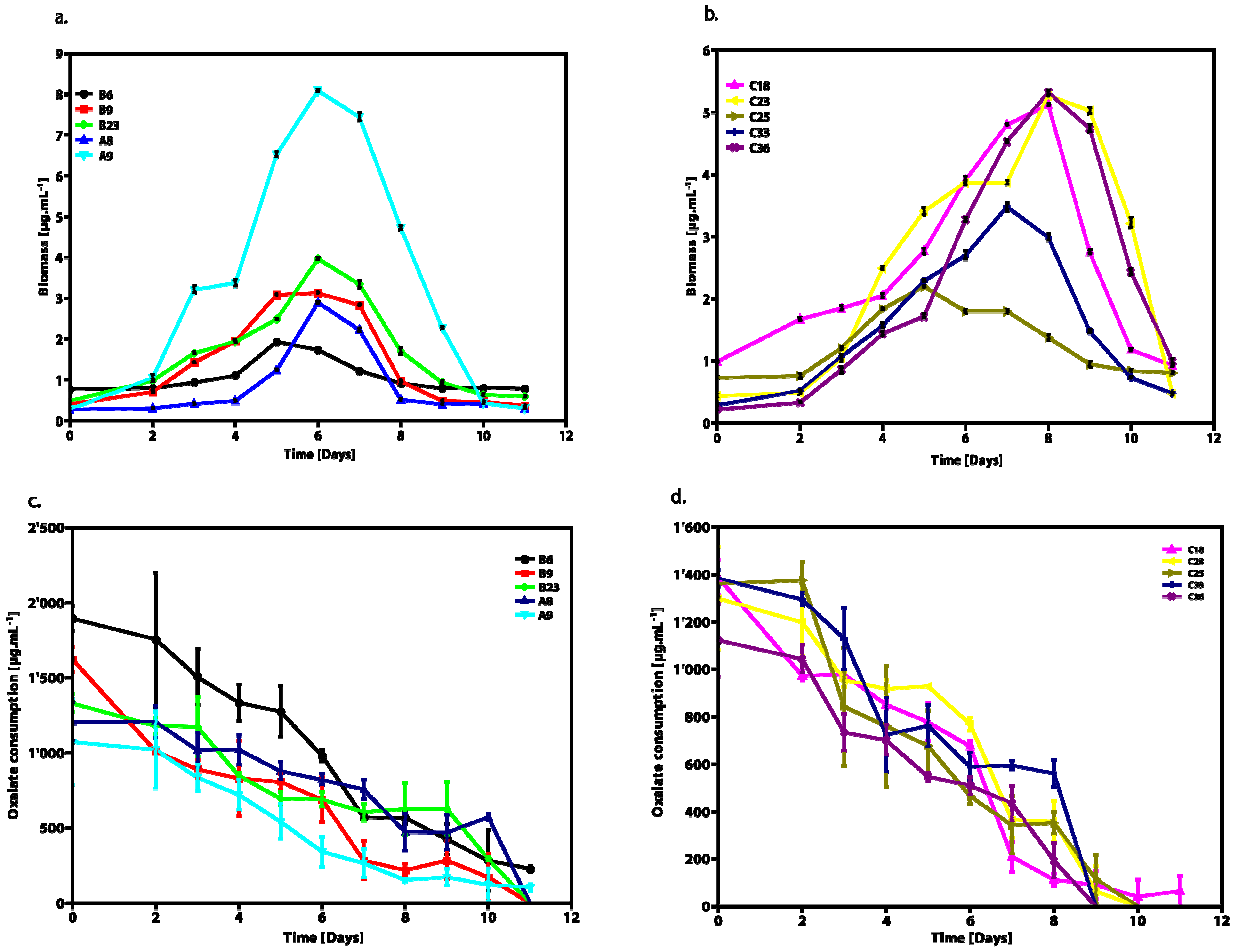


Figure 2. Comparison of growth measured as total proteins (Biomass in µg.mL^{-1}), and oxalate consumption (µg . mL^{-1}) for ten oxalotrophic bacterial strains from Bolivia, India, and Cameroon. (**a.**) Comparison of growth curves between Bolivian and Indian strains. (**b.**) Comparison of growth curves within Cameroon strains. (**c.**) Oxalate degradation for Bolivian and Indian strains. (**d.**) Oxalate degradation obtained for Cameroon strains. B6 = *Bacillus* sp.; B9 = *Xhantomonas* sp.; B23 = *Variovorax* sp.; A8 = *Lysobacter* sp.; A9 = *Streptomyces achromogenes*; C18 = *Variovorax soli*; C23 = *Agrobacterium* sp.; C25 = *Paenibacillus* sp.; C33 = *Stenotrophomonas maltophilia*; C36 = *Stenotrophomonas* sp. The consumption of oxalate was measured by HPLC at the same time when growth was assessed.

iv. Oxalotrophic activity measured by IMC

A microcalorimetric assay was performed to obtain kinetic parameters of growth from the selected oxalotrophic strains. Representative thermograms are shown in Figure 4. After 170 h of incubation at 25±1°C, no change in both baseline and negative control (*E. coli* K12) signals was observed. In contrast, in all strains selected a clear peak of activity was observed. The parameters of biological activity derived from the heat flow (Table 3) showed that the strain *V. soli* C18 has the highest consumption rate growing with Kox, with 0.240 μM h^{-1}. This strain was followed by *Lysobacter* sp. A8 and *Agrobacterium* sp. C23, with 0.221 and 0.209 μM h^{-1}, respectively.

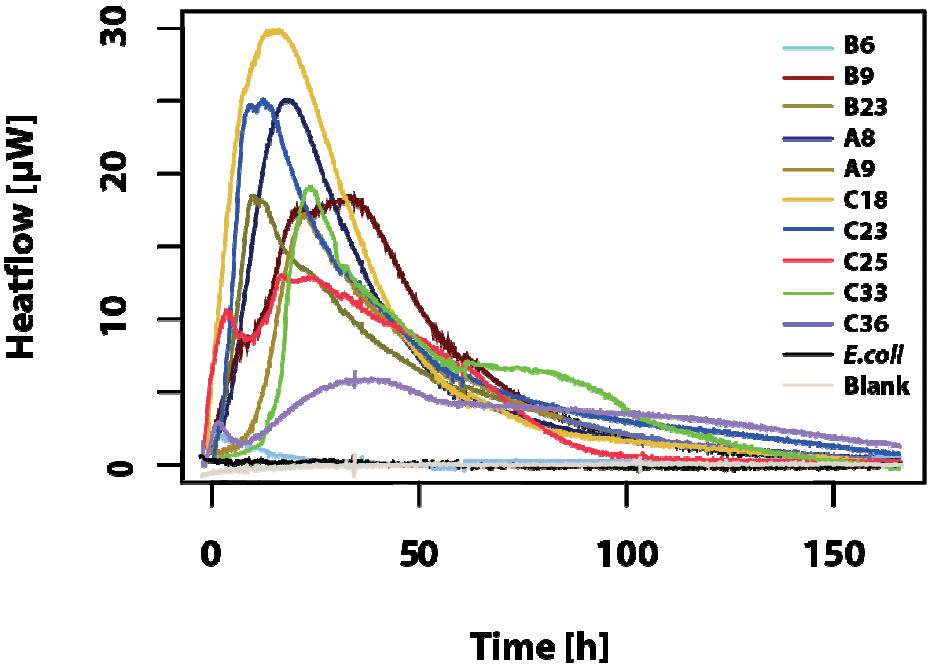


Figure 3. Representative IMC thermograms recorded to measure oxalotrophic activity in ten selected oxalotrophic strains growing with Angle medium supplemented with potassium oxalate (Kox). The baseline (Blank) consisted of sterile Angle medium with Kox. *Escherichia coli* K12 (*E.coli*) was used as negative control. For the identity of the strains see Table 2. The thermogram shows the strain *Variovorax soli* C18 with a maximal heat-flow of 30.3 μW and the highest consumption rate (0.240 $\mu M.h^{-1}$).

Table 3. Kinetic parameters obtained after the analysis of heat flow thermograms measured for ten selected oxalotrophic strains growing in Angle medium with potasium oxalate. *Escherichia coli* K12 was used as negative control. Blanks consisted in sterile culture medium. ND= not determined. n= number of replicates.

Strains	Maximum heatflow [µW]	Oxalate consumption rate [µmol.h^{-1}]	growth rate [h^{-1}]	n
Bacillus sp. (B6)	3,273 ± 0,985	0,024 ± 0,007	0,012 ± 0,010	4
Xanthomonas sp. (B9)	22,676 ± 0,772	0,164 ± 0,006	0,065 ± 0,012	4
Variovorax sp. (B23)	25,742 ± 1,840	0,186 ± 0,013	0,155 ± 0,023	4
Lysobacter sp. (A8)	30,639 ± 1,057	0,221 ± 0,008	0,124 ± 0,019	4
Streptomyces achromogenes (A9)	20,821 ± 1,260	0,150 ± 0,009	0,171 ± 0,006	4
Variovorax soli (C18)	33,276 ± 2,099	0,240 ± 0,015	0,085 ± 0,007	4
Agrobacterium sp. (C23)	28,975 ± 0,904	0,209 ± 0,007	0,228 ± 0,018	4
Paenibacillus sp. (C25)	16,156 ± 1,466	0,117 ± 0,011	0,036 ± 0,033	4
Stenotrophomonas maltophilia (C33)	22,974 ± 2,756	0,166 ± 0,020	0,115 ± 0,010	4
Stenotrophomonas sp. (C36)	10,269 ± 0,402	0,074 ± 0,003	0,051 ± 0,005	4
Escherichia coli K12	ND	ND	ND	3
Blanks	ND	ND	ND	2

v. Metabolic profiles of oxalotrophic bacteria by BIOLOG

The metabolic diversity of the selected oxalotrophic strains was measured as well (Table 4). Overall, there was a greater utilization of a number of polymers and carbohydrates and in less proportion of amino acids, amines or even other carbon sources (e.g. alcohols). The biochemical test employed (BIOLOG Microplate SN-F2) suggested a high metabolic plasticity for the strain *S. achromogenes* A9 (Table 4), which assimilated five polymeric substances (α-ciclohexine, Dextrin, Glycogen, Tween 40 and Tween 80), ten carbohydrates (e.g. N-Acetyl, D-Glucosamine, or D-Trehalose), and two amino acids (L-Glutamic acid and Aspartic acid). A second strain capable of consuming several carbon substrates was *Agrobacterium* sp. C23 (Table 4). This strain was able to degrade α-Cyclodextrin, consumed seven carbohydrates (e.g. N-Acetyl, D-Glucosamine or D-raffinose), grew in glycerol, and used the same amino acids as *S. achromogenes* A9. *Lysobacter* sp. A8 also showed a large metabolic plasticity. It was able to degrade, for instance, the same polymers as *Agrobacterium* sp. C23, but besides, it grew in Tween 40, and consumed seven carbohydrates (from Adonitol to Manitol). The strain *S. maltophilia* C33 was able to use 6 carbohydrates (e.g. N-Acetyl D-Glucosamine and D-Raffinose), and the strain *Variovorax* sp. B23 was able to degrade α-Cyclodextrin, and to consume 3 carbohydrates (L-Arabinose, D-Mannose, and D-Melibiose), as well as the amine 2-Aminoethanol. Finally, the strains *Bacillus* sp. B6, *Xanthomonas* sp. B9, *Variovorax* sp. C18, and *Paenibacillus* sp. C25 showed a limited plasticity with only two carbon sources consumed. Interestingly, *Paenibacillus* sp. was the only strain able to consume the

carboxylic acid poly-hydroxy phenyl acetic acid. The strain *Stenotrophomonas* sp. C36 grew only in L-Arabinose as carbon source, besides Caox and Kox.

Table 4. Biochemical characterization of selected oxalotrophic strains using the Biolog system SF-N2 MicroPlate™. The strains were incubated at room temperature during 7 days. Only substrates used by at least one strain are included in the table.

Substrate	B6	B9	B23	A8	A9	C18	C23	C25	C33	C36
α-Cyclodextrin			+	+	+		+			
Dextrin					+					
Glycogen	+				+					
Tween 40				+	+					
Tween 80					+					
N-Acetyl-D-Galactosamine										
N-Acetyl-D-Glucosamine					+		+		+	
Adonitol				+						
L-Arabinose		+	+	+	+	+	+	+	+	+
D-Arabitol				+	+					
D-Cellobiose				+						
D-Fructose				+						
α-D-Glucose				+	+	+			+	
D-Mannitol				+	+					
Succinic acid	+									
D-Mannose		+	+							
D-Melibiose			+				+			
2-Aminoethanol			+							
L-Fucose					+		+			
D-Galactose					+				+	
D-Lactose					+					
L-Rhamnose					+					
D-Trehalose					+		+			
L-Glutamic acid					+		+			
Aspartic acid					+		+			
D-Gentibiose							+		+	
D-Raffinose							+		+	
Glycerol							+			
Poly-hydroxy phenyl acetic acid								+		

d. Discussion

i. Tropical forest and oxalotrophic bacteria

Previous studies in tropical forest with Iroko trees in Cameroon (Cailleau, et al., 2011) and in Ivory Coast (Cailleau, et al., 2004) evaluated the capacity of plants to enhance mineral carbon accumulation, and how the coupled biogeochemical cycles of carbon and calcium lead to the storage of inorganic carbon (Braissant, et al., 2004). However, prokaryotes are recognized as the main responsible for the mineralization of plant biomass (Schlegel, 1986, Gunnarsson, et al., 1988, Zech, et al., 1997), especially in tropical soils (Zech, et al., 1997), and therefore, the assessment of the diversity of oxalotrophic bacteria in this study represents a first step towards the understanding of their contribution to different OCP tropical ecosystems. This study showed that conventional culturing methods allow the recovery of a diverse assemblage of oxalotrophic bacteria living in the rhizosphere of oxalogenic trees in tropical forest from Bolivia, India, and Cameroon.

In addition, it confirms that an active OCP (the combination of the presence of an oxalogenic tree, soil alkalinization and oxalotrophic microorganisms) can be found outside the African continent. As in the case of the Iroko, the two newly recognized oxalogenic trees, *T. oblonga* and *T. bellirica*, are relevant in agroforestry, which is an added value of the OCP. For example, *T. oblonga* (Bolivia) has been considered as an important species for conservation and management of tropical forest in lowland South America as it regenerates vigorously (Mostacedo C & Fredericksen, 1999) and shows favorable surviving rates (Schiøtz, et al., 2006). Likewise, T. bellirica, a perennial deciduous tropical tree found in South Asia (Reddy, et al., 2011), has been reported as economically important due to its medical properties (Phulwaria, et al., 2012), such as the production of pharmaceuticals (e.g. anti-stressor, anti-oxidant, and immune-stimulant) from fruit extracts (Srikumar, et al., 2006).

Based on the results of culturing, there were significant differences in the recovery of oxalotrophic bacteria from soils in the three tropical sites studied. In Bolivia, it was possible to isolate oxalotrophic bacteria from both soil profiles, while in India and Cameroon oxalotrophic bacteria were only isolated from soils collected near the oxalogenic tree. These results are relevant for two reasons. First, the distribution of oxalotrophs might reflect the effect of tree-specific rhizospheric influence on substrate availability (i.e. rate of oxalate release), which is likely affecting abundance as shown by a larger recovery of oxalotrophs in the top five centimeters. This area corresponds to the zone in which the Caox-rich plant organic matter and saprophytic

activity is enhanced (Ström, et al., 2005). Second, the structure of the forest could also have an influence since, in the case of Bolivia, a dense vegetation cover (Supplementary Fig. 1a) made harder to define a soil not influenced by a Caox flux from *T. oblonga* or other potential oxalogenic trees. In contrast, in India and Cameroon (Supplementary Fig. 1b and 1c, respectively), the standing oxalogenic trees were relatively isolated making less likely other Caox sources into the soil. This last aspect, a spatial distribution pattern associated to Caox release, is surprising considering that most oxalotrophic bacteria have been considered generalists and hence ubiquitous (Sahin, 2003). This will be considered further in the analysis of the metabolic plasticity of the strains characterized in the present study.

ii. Diversity of oxalotrophic bacteria

In the present work, 95 phylogenetically diverse oxalotrophic bacteria were isolated from three tropical habitats. A relatively high diversity, including strains related to Alpha-, Beta-, and Gamma- Proteobacteria, Firmicutes, and Actinobacteria was observed. Alpha, Beta, and Gamma-Proteobacteria correspond to a large fraction of the known culturable oxalotrophic diversity (Sahin, et al., 2002, Tamer, et al., 2002, Sahin, 2003, Sahin, et al., 2008). Oxalotrophic activity has been also reported in previous studies in Firmicutes (Turroni, et al., 2007), Actinobacteria (Müller, 1950, Knutson, et al., 1980, Sahin, 2004), and diazotrophic bacteria (Trinchant & Rigaud, 1996, Sahin, 2005). Therefore, the diversity of oxalotrophic bacteria identified in the present study, was spread over all mentioned cases. Moreover, the present study reports for the first time strains related to the genera *Sphingomonas*, *Afipia*, *Rhodospirillum*, *Polaromonas*, *Terrabacter*, and *Ensifer* as oxalotrophic bacteria found in tropical soils from India and Cameroon. This diversity shows that oxalate is an important carbon source for specific rhizospheric non-related bacterial groups in forest soils, as has been suggested previously (Sahin, 2005). This pool of oxalotrophic bacteria constitutes a valuable collection, since it represents a large number of the culturable groups of this poorly known functional group. Among the 95 strains isolated, 10 were characterized based on their oxalotrophic activity and metabolic plasticity. The relevance of each one of them is discussed in the following section.

iii. Characterization of selected oxalotrophic strains and their potential role in the OCP

Almost all the work performed so far in oxalotrophy has been carried out with model strains, such as *Cupriavidus oxalaticus* (Quayle, et al., 1961, Dijkhuizen, et al., 1977, Schneider, et al., 2012), *Methylobacterium extorquens* (Guo & Lidstrom, 2008), *Oxalicibacterium flavum* (Tamer, et al., 2002), or the medically relevant species *Oxalobacter formigenes*. Other studies have been carried out with anaerobic oxalotrophs in beech forest soils (Daniel, et al., 2007). Nevertheless, this is the first time that environmental oxalotrophic aerobic strains directly related with the OCP, are assessed to understand their metabolic capability to catabolize oxalate as carbon and - or energy source.

In soils, oxalate is one of the most common carbon sources available, especially in litter, and its utilization by microorganisms is a response to long-term carbon input provided in the rhizosphere (Brant, et al., 2006). Previous studies performed in forest soils (van Hees, et al., 2002) showed that the rates of microbial decomposition of low molecular weight organic acids (such as oxalate) expressed as maximum mineralization rate of oxalate (Vmax) are close from those measured for *V. soli* C18, *Lysobacter* sp. A8, and *Agrobacterium* sp. C23 in this study. Indeed, the parameters measured in this study are directly related with the dynamics of the carbon storage in soil, and thus, the dynamics of the oxalate-carbonate pathway in a global perspective.

The differences observed between growth and consumption of oxalate for the selected strains could give indications that not all strains used oxalate as carbon, but rather as energy source (Quayle & Keech, 1960, Blackmore & Quayle, 1968). Therefore, environmental non phylogenetically related strains may relay on several pathways at different levels for the oxidation of oxalate, such has been demonstrated with model strains (Quayle, et al., 1961). This is suggested by the results of the characterization in the present study. For example, *Bacillus* sp. B6 has shown a maximum biomass production at 5 days of incubation whereas other strains with greater biomass production, such as *S. achromogenes* A9 (Fig. 3), and greater oxalotrophic activity such as *Variovorax soli* C18 have shown a maximal biomass production later on (between 6 and 8 days, respectively). Therefore, the time frame of maximum growth could be related with a diversity of metabolic pathways (energy utilization) activated between oxalotrophs isolated from the OCP systems. In this sense, the results obtained with the selected environmental strains may confirm the idea that oxalotrophic bacteria do not necessary use oxalate for growth, but for biosynthesis purposes (as energy source), as in the case of the glyoxylate pathway (Wagner & Quayle, 1972), or amino acids synthesis pathways such as the serine-glycine and threonine pathways (Friedrich, et al., 1979).

Two morphologically distinct Bacilli strains, *Bacillus* sp. B6 and *Paenibacillus* sp. C25, were characterized. Although, both solubilized Caox in solid medium, they grew deficiently in liquid medium. The results of the kinetic characterization were confirmed by IMC showing the lowest growth rate (0.012 and 0.036 h^{-1}). Also, the Bacilli strains studied here showed lesser metabolic capability compared to the other strains. Interestingly, only *Bacillus* sp. B6 was unable to consume arabinose (Table 4). Arabinose is present in hemicellulose and pectin, i.e. component of the plant cell wall (Burget, et al., 2003). Moreover, it has been demonstrated that arabinose is a precursor for oxalic acid formation (Loewus, et al., 1995), and actinobacteria are able to consume this substrate (Qin, et al., 2010). The role of Arabinose as intermediary in oxalate degradation should be taken into account for further metabolomics studies using the strains isolated here. The low metabolic activity and plasticity of Bacilli suggest that their involvement in the OCP is not as Caox consumers. For example, strains related to the genus *Paenibacillus* (e.g. *Paenibacillus kribbensis*) have been shown to produce oxalic acid for the solubilization of complex inorganic calcium and iron phosphates (Marra, et al., 2012), and therefore those bacteria can rather participate to the production than the consumption of oxalate.

The strain *Xanthomonas* sp. B9 was related *to Ensifer adhaerens* and *Rhodospirillum* sp. strains that have been isolated in a minor proportion in Cameroon soil samples. One feature of this strain was colony pigmentation as has been previously reported for other strains belonging to the genus. *Xanthomonas* sp. B9 has shown an important degradation halo in solid Schlegel AB+Caox. The growth kinetics and IMC analysis confirmed the capability of this strain to consume oxalate. *Xanthomonas* are reported primarily as plant pathogens responsible for necrotic lesions (Starr & Stephens, 1964). Although this genus has a large metabolic plasticity, and therefore has been suggested for bioremediation in terrestrial polluted environments (Trower, et al., 1985), the BIOLOG profile of *Xanthomonas* sp. B9 showed a poor capability to degrade carbon sources other than Caox. Thus *Xanthomonas* strains in the OCP could be a group of oxalotrophic bacteria with low metabolic plasticity and high oxalate consumption capacity. The fact that the strain B9 was isolated from the upper part of the profile A, could be related with an important release of Caox in the litter, an ideal place for Caox growth-dependent bacteria.

The genus *Variovorax* was represented by two closely related strains: *Variovorax* sp. B23 and *V. soli* C18. This genus has been recognized to be one of the most important groups in rhizospheric soils (Jamieson et al. 2009). Both strains grew well and consume Caox in similar way. In IMC, these strains showed a high growth rate. Their maximum consumption rate was considerably higher compared with strains discussed before, especially for *V. soli* sp. B23, which showed the

highest heat-flow (33.27 μW, Fig. 4). The BIOLOG profile showed that both used a limited range of carbon sources. Previous studies have shown that *Variovorax paradoxus*, an oxalotrophic model (strain DSM 30034), is one of the most persistent oxalotrophic bacteria found in microcosm soils (Martin, et al., 2012). Therefore *V. soli* C18 could be fit in the role of efficient oxalotrophic bacteria implied in the OCP system from Cameroon.

The strain *Lysobacter* sp. A8 was phylogenetically related to *Xanthomonas*, as part of the class Gamma-Proteobacteria. Members of this genus are known to have high metabolic rates in soil, for several complex substrates. The strain *Lysobacter* sp. A8 produced less biomass compared to others like *Variovorax* sp. B23 or *Streptomyces* sp. A9; but was able to consume and grow on oxalate. This strain had one of the higher consumption rates of oxalate with 0.221 μM h^{-1} of Kox. Closely related members of the genus *Lysobacter* may act as regulators of bacterial competitors to use oxalate, since they are known to have lysogenic activity (Ahmed, et al., 2003). The BIOLOG profile showed a more versatile metabolism compared to the precedent strains. The consumption of polymers could be related with their ecological importance, thus, used as biocontrol agent against plant fungal pathogens (Aslam, et al., 2009). In the OCP, this strain could be relevant at the phyllosphere level, since their lysogenic capacity will enhance the release of carbon sources such as oxalate crystals.

The group of *Streptomyces* probably is the less studied, but is likely one of the most important in the OCP occurring in tropical soils. Bacilli and Actinobacteria were the two Gram-positive oxalotrophs isolated in this study. Streptomyces sp. was the most represented genus. The strain *S. achromogenes* A9 was isolated at the litter layer of the profile A in India. Biomass production for *S. achromogenes* A9 in liquid medium with Caox was the highest obtained (8 μg mL^{-1}). Recently, it has been demonstrated that both model and environmental strains of *Streptomyces* consume important amounts of Caox (Bravo, et al., 2011). In this study, *S. achromogenes* A9 was able to consume oxalate, producing net and maximal growth rates higher than all other strains tested (Fig. 4). *Streptomyces* spp. have been recognized as colonizers due to their filament morphology (Goodfellow & Williams, 1983), as well as saprophytes (Sahin, 2004). According to this description, the strain *S. achromogenes* A9 showed the higher metabolic plasticity in the BIOLOG profile. Therefore, their function in soil as potential oxalate releasers and consumers make this group a versatile and crucial player in the OCP from tropical ecosystems. Since a long time, the group of actinobacteria has been reported as oxalotrophic in soil or litter (Müller, 1950, Knutson, et al., 1980, Messini & Favilli, 1990, Sahin, 2003, Sahin, 2004), but only a few studies have pointed out to this group in the context of the OCP (Braissant, et al., 2004). Therefore, this

group could be relevant regarding its presence in the three OCP systems studied. *Streptomyces* could be implicated in the dispersion of oxalate in soil and litter, due their filamentous morphology, as seen by the isolation of *Streptomyces* from the control soil in Bolivia (*Streptomyces* sp. B14 found 10 m away from the tree).

Agrobacterium sp. C23 was isolated from Cameroon. This genus is related with cancer disease in several plants (Gelvin, 2003). The kinetic growth showed that this strain grew well in medium with Caox as sole carbon source (5.3 μg mL^{-1} of biomass). IMC data showed a high thermal signal for Kox (growth rate of 0.228 h^{-1}, and oxalate consumption rate of 0.209 μM h^{-1} of Kox). This strain showed the second maximum consumption rate of Kox after *V. soli* C18. The BIOLOG profile showed that the strain *Agrobacterium* sp. has a high metabolic plasticity. Its presence could be related to the flux of nitrogen fixation in soil, contributing to the efficiency of oxalate oxidation.

Finally, the genus *Stenotrophomonas* was represented by two strains, *S. maltophilia* C33 and *Stenotrophomonas* sp. C36, both from Cameroon soil samples. This genus occurs ubiquitously in the environment, but especially in soil and plants (Ryan, et al., 2009), and it is recognized to have a beneficial interaction with plants, since no *Stenotrophomonas* species are known to be phytopathogenic (Ryan, et al., 2009). *S. maltophilia* is a dominant member of the rhizospheric microbial community (Juhnke & des Jardin, 1989). Kinetic curves showed that *S. maltophilia* C33 grew less than *Stenotrophomonas* sp. C36 in Caox (3.4 and 5.4 μg mL^{-1} of biomass). IMC showed that *S. maltophilia* C33 grew with a maximum heat-flow of 22.97 μW, instead of this, *Stenotrophomonas* sp. C36 showed a maximum heat-flow of 10.29 μW. Thus, the maximum consumption rates were proportional to the heat-flow (0.166 and 0.074 μM h^{-1} of Kox for the strains C33 and C36, respectively). The BIOLOG profile confirmed the metabolic capacities of those strains. *S. maltophilia* C33 have a higher metabolic capacity in comparison with *Stenotrophomonas* sp. C36 (only one substrate consumed, L-Arabinose), degrading α-cyclodextrin, and six carbohydrates (Table 4), indicating that morphological differences, but also metabolic diversity, could be found in environmental oxalotrophic *Stenotrophomonas* involved in the OCP. The abundance of this genus was highlighted in Bolivian soil samples, and even if they represented half of the total isolated strains, their metabolic capability to oxidize Caox or other oxalate salts released in soil, might not be the same.

e. Conclusion

We have shown that the diversity of culturable oxalotrophic bacteria presented in a field effort on three continents improved the overall knowledge of this key functional group in the oxalate-carbonate pathway. Interestingly, a large fraction of the oxalotrophic bacteria isolated here corresponded to rhizospheric bacteria that may play a role in long-term soil pedogenic processes, such as the OCP. The results obtained in this study showed that strains such as *V. soli* C18, *Lysobacter* sp. A8, and *Agrobacterium* sp. C23 were the most metabolic active oxalotrophs tested in OCP systems from India and Cameroon, while strains such as *S. maltophilia* seemed to represent an important fraction of the oxalotrophs in the Bolivian OCP system. Moreover, the capabilities for the utilization of other carbon substrates suggest that *S. achromogenes* A9 is the most versatile oxalotroph. Further studies combining culturable and non-culturable methods, as well the mineralization kinetics to quantify the contribution of oxalotrophs in the biomineralization of oxalate into secondary carbonate, as well as experimental plant-soil atmosphere system (ESPAS) devices, represent next steps in studies dealing with the role of bacteria in the OCP.

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h. Supplementary tables

Supplementary Table 1. Oxalotrophic bacterial isolates from Bolivia, obtained from control soils (Profile C) and soil sample at one side to the oxalogenic tree (Profile A). Selected strains for the physiological and metabolic characterizations are shown in bold.

Strain	Colony description	Profile	Depth [cm]	Halos [cm]
B1	Translucent, cremose, flat border, small	C	0-10	0.2
B2	White, irregular border, concave, small, powdery	C	0-10	0.2
B3	White, flat border, flat, small, punctiform and viscous	C	0-10	0.3
B4	Brown, flat border, small, punctiform, dull	C	0-10	0.2
B5	White, small, mucoid, punctiform	C	0-10	0.4
B6	Brown, small, mucoid	C	0-10	0.5
B7	Translucent, irregular border, punctiform & consistent	C	0-10	0.2
B8	Opaque, flat border, median, punctiform	C	0-10	0.1
B9	Yellow-brown, irregular border, small, punctiform and powdery	C	0-10	0.6
B10	White, flat border, punctiform, small	C	74-76	0.3
B11	Cream, diminutes, punctiform	C	74-76	0.4
B12	Gray, punctiform, powdery, small	C	74-76	0.3
B14	Brown, powdery, concentric growth	C	74-76	0.2
B15	Cream, small, punctiform	A	0-10	0.1
B16	Cream, irregular border, dull	A	0-10	0.2
B17	Yellow, creamy, regular border, dull	A	0-10	0.1
B19	Cream, punctiform, irregular border	A	0-10	0.1
B20	Dull, irregular border, creamy	A	0-10	0.2
B21	Brown-white, punctiform, median, creamy	A	0-10	0.3
B22	Brown, powdery, punctiform, small	A	0-10	0.4
B23	Brown, irregular border, diminutes, consistent, small points	A	0-10	0.7
B24	Brown, powdery, punctiform	A	0-10	0.3
B25	Brown, median, powdery, concentric spirals	A	74-76	0.2
B26	Brown, punctiform, diminutes	A	74-76	0.2
B27	Cream, regular border, dull	A	74-76	0.1
B28	Cream, dull, punctiform, median & large concentric spirals	A	74-76	0.1
B29	Brown, powdery, diminutes, punctiform	A	74-76	0.2
B31	Brown, powdery, small, punctiform	A	74-76	0.3
B32	Brown, diminutes, punctiform	A	74-76	0.4

Supplementary Table 1 continuation. Oxalotrophic bacterial isolates from India, obtained from control soils (Profile C) and soil sample at one side to the oxalogenic tree (Profile A). Oxalotrophy = time for the observation of a halo on solid Schlegel medium. Selected strains for the physiological and metabolic characterizations are shown in bold.

Strain	Colony description	Profile	Depth [cm]	Halos [cm]
A1	Gray-white, powdery, punctiform	A	0-1	0.1
A2	Brown, irregular border, powdery, punctiform, small	A	0-1	0.3
A5	Cream, irregular border, powdery, halo dull, punctiform, small	A	0-1	0.2
A7	Gray, irregular border, dull, punctiform, concentric and opaque	A	0-1	0.2
A8	Opaque centric, median	A	0-1	0.7
A9	Brown, irregular border, halo dull, double ring, powdery, small	A	0-1	0.8
A10	Cream, regular border, flat, opaque, punctiform, big colonies	A	0-1	0.2
A12	White, punctiform, small, opaque	A	0-1	0.1
A13	Brown, powdery, opaque, punctiform	A	0-1	0.3
A14	Cream, irregular border, powdery, punctiform	A	1-2	0.3
A16	Gray-blue, irregular border, concentric, dull, punctiform	A	1-2	0.4
A21	White, irregular border, powdery, circular colonies	A	1-2	0.2
A22	Brown, opaque, irregular border, circular growth	A	2-3	0.3
A23	White, powdery, irregular border, median, swarming	A	2-3	0.1
A27	White, small, halo opaque dull centre compacted	A	2-3	0.1
A28	White, irregular halo opaque, powdery, median	A	2-3	0.1
A29	White, irregular, median, opaque, powdery	A	2-3	0.1
A31	Brown irregular, powdery, small, swarming colonies	A	3-4	0.2
A32	Gray, opaque, irregular border, swarming	A	3-4	0.3
A33	Brown, irregular border, small, powdery, swarming	A	4-5	0.4
A34	Brown to green, very powdery, irregular border, big	A	4-5	0.4
A35	Brown, powdery, median, opaque, swarming	A	4-5	0.3
A36	Brown, powdery, punctiform, median, opaque, swarming	A	4-5	0.2
A38	Grey-blue, median size, powdery, opaque, irregular border	A	5-10	0.1
A43	White, punctiform, irregular border, opaque, median size	A	50	0.1
A46	White, very swarming, median, opaque, punctiform	A	50-55	0.1
A55	Brilliant, cream, regular border, concave, median, ovoid	A	101-102	0.2

Supplementary Table 1 continuation. Oxalotrophic bacterial isolates from Cameroon, obtained from soil sample at one side to the oxalogenic tree (Profile A). Oxalotrophy = time for the observation of a halo on solid Schlegel medium. Selected strains for the physiological and metabolic characterizations are shown in bold.

Strain	Colony description	Profile	Depth [cm]	Halos [cm]
C1	Gray, powdery, punctiform, small	A	1-5	0.4
C3	Gray, irregular border, powdery, punctiform	A	1-5	0.3
C4	Gray, diminutes, powdery	A	1-5	0.4
C5	Brown, median, powdery, concentric spirals	A	1-5	0.3
C6	Brown, diminutes, punctiform, irregular border, powdery	A	1-5	0.3
C7	Cream, punctiform & dull	A	6-8	0.2
C8	Gray, small, punctiform & powdery	A	6-8	0.4
C9	Cream, diminutes, punctiform	A	6-8	0.1
C10	Cream, flat border, punctiform, small, creamy	A	6-8	0.1
C11	Orange, median, irregular border, circular	A	10-11	0.2
C12	Brown, punctiform, powdery	A	10-11	0.2
C13	Cream, punctiform, dull	A	10-11	0.1
C14	Cream, translucent, small, dull	A	10-11	0.1
C15	Cream, median, irregular border, creamy	A	12-14	0.2
C16	Cream, flat border, punctiform, median	A	12-14	0.1
C17	Cream, translucent	A	12-14	0.1
C18	Cream, translucent, concentric spirals, dull, irregular border	A	12-14	0.8
C19	Cream, concentric spirals, irregular border	A	12-14	0.2
C20	Cream, irregular border	A	12-14	0.1
C21	White, cream	A	16-17	0.2
C22	Cream, dull, creamy	A	16-17	0.2
C23	Gray-Brown, diminutes, powdery	A	16-17	1.3
C24	Cream, small, creamy	A	16-17	0.1
C25	Cream, translucent, irregular border, median	A	30-32	0.5
C26	Brown, powdery, small, dull	A	30-32	0.4
C27	Brown, irregular border, dull, powdery	A	30-32	0.4
C28	Orange, big, irregular border, concentric spirals	A	32-33	0.3
C29	Cream, punctiform, dull	A	32-33	0.2
C30	Cream, diminutes, dull	A	32-33	0.1
C31	Translucent, punctiform, dull, creamy	A	32-33	0.1
C32	Orange, median, irregular border, circular	A	32-33	0.2
C33	Brown, powdery, small, dull	A	45-46	0.7
C34	Gray, creamy, punctiform, irregular border	A	45-46	0.3
C35	Gray, powdery, punctiform, dull	A	45-46	0.3
C36	Gray, powdery, punctiform, small, irregular border	A	45-46	0.6
C37	Gray, punctiform, small, concentric spirals, dull, powdery	A	55-56	0.4
C38	Cream, punctiform, creamy, dull	A	55-56	0.2
C39	Translucent, creamy, dull	A	55-56	0.1
C40	Gray, small, powdery, diminutes	A	55-56	0.4

Supplementary Table 2. Results of BLASTn obtained after partial sequencing of the 16S rRNA gene (1000 bps). The primers used correspond to the universal primers Eub9_27 forward and Eub1542 reverse (Liesack*, et al.*, 1991). The sequences were aligned with muscle using Mega 5 (Tamura*, et al.*, 2011).

Bolivia

Strain	Name	ID BLAST	Accession no.
B1	*Stenotrophomonas maltophilia*	99	AB683956.1
B2	*Stenotrophomonas maltophilia*	95	AB683956.1
B3	*Stenotrophomonas maltophilia*	93	NC_015947
B4	*Stenotrophomonas maltophilia*	98	AB683956.1
B5	*Stenotrophomonas maltophilia*	98	AB683956.1
B6	*Bacillus* sp.	97	AM900775.1
B7	*Stenotrophomonas maltophilia*	98	CP002986.1
B8	*Stenotrophomonas maltophilia*	98	AGRB01000147.1
B9	*Xanthomonas* sp.	99	HQ891021.1
B10	*Stenotrophomonas maltophilia*	99	AB683956.1
B11	*Acidovorax* sp.	85	HM060199.1
B12	*Stenotrophomonas maltophilia*	99	AB683956.1
B14	*Streptomyces fungicidicus*	97	JN368424.1
B15	*Streptomyces* sp.	85	HM060199.1
B16	*Xanthomonas campestris*	96	NC_010688
B17	*Xanthomonas campestris*	99	JN652252.1
B19	*Stenotrophomonas* sp.	98	EF219038.1
B20	*Stenotrophomonas maltophilia*	92	JF330158.1
B21	*Stenotrophomonas maltophilia*	93	AB683956.1
B22	*Agrobacterium* sp.	85	EU073111.1
B23	*Variovorax* sp.	96	JQ010855.1
B24	*Streptomyces griseus*	95	EF154278.1
B25	*Stenotrophomonas maltophilia*	99	AB683956.1
B26	*Stenotrophomonas maltophilia*	96	AB683956.1
B27	*Stenotrophomonas maltophilia*	96	HQ434490.1
B28	*Streptomyces* sp.	95	JF806661.1
B29	*Streptomyces sp.*	94	HQ850380.1
B31	*Streptomyces* sp.	97	JF806661.1
B32	*Streptomyces* sp.	91	JN866724.1

Supplementary Table 2 continuation.

India

Strain	Name	ID BLAST	Accession no.
A1	*Streptomyces* sp.	95	EF527811.1
A2	*Streptomyces* sp.	99	JN969032.1
A5	*Paenibacillus* sp.	98	CP001793.1
A7	*Lysobacter* sp.	95	FR667176.1
A8	*Lysobacter* sp.	99	FR667176.1
A9	*Streptomyces achromogenes*	98	JN400102.1
A10	*Streptomyces* sp.	99	HQ662221.1
A12	*Streptomyces hygroscopicus*	94	JF439586.1
A13	*Paenibacillus* sp.	99	EU621913.1
A14	*Streptomyces purpurascens*	97	JF895538.1
A16	*Paenibacillus glucanolyticus*	97	AB680838.1
A21	*Streptomyces purpurascens*	78	AJ781382.1
A22	*Paenibacillus* sp.	98	FJ233853.1
A23	*Streptomyces purpurascens*	92	AJ781382.1
A27	*Afipia* sp.	99	AB586143.1
A28	*Streptomyces purpurascens*	98	AJ781382.1
A29	*Paenibacillus* sp.	96	FJ233853.1
A31	*Streptomyces* sp.	77	JF830630.1
A32	*Paenibacillus glucanolyticus*	82	AB680838.1
A33	*Afipia broomeae*	97	AY568506.2
A34	*Sphingomonas* sp.	99	HE962513.1
A35	*Polaromonas jejeuensis*	84	AB682445.1
A36	*Bacillus psychrodurans*	96	JF970581.1
A38	*Streptomyces* sp.	98	GQ340692.2
A43	*Paenibacillus* sp.	89	CP001793.1
A46	*Terrabacter* sp.	99	JQ958845.1
A55	*Rhizobium giardinii*	96	JQ342849.1

Supplementary Table 2 continuation.

Cameroon

Strain	Name	ID BLAST	Accession no.
C1	*Ralstonia* sp.	98	HM233962.1
C3	*Stenotrophomonas* sp.	99	JQ917792.1
C4	*Stenotrophomonas maltophilia*	95	HQ434490.1
C5	*Achromobacter* sp.	98	JQ746496.1
C6	*Sinorhizobium* sp.	98	FR749836.1
C7	*Variovorax* sp.	99	HQ730968.1
C8	*Streptomyces* sp.	86	JN866688.1
C9	*Agrobacterium* sp.	99	JF730141.1
C10	*Stenotrophomonas maltophilia*	94	JF330158.1
C11	*Variovorax* sp.	99	HQ730968.1
C12	*Variovorax soli*	99	AB682436.1
C13	*Sinorhizobium* sp.	89	HQ290085.1
C14	*Achromobacter* sp.	99	JQ746498.1
C15	*Stenotrophomonas maltophilia*	99	HM584272.1
C16	*Agrobacterium* sp.	99	JF730141.1
C17	*Ensifer adhaerens*	92	DQ140416.1
C18	*Variovorax soli*	89	AB682436.1
C19	*Cupriavidus respiraculi*	84	JF682073.1
C20	*Achromobacter* sp.	97	FN794206.1
C21	*Arthrobacter* sp.	97	DQ847127.1
C22	*Stenotrophomonas maltophilia*	97	AB683956.1
C23	*Agrobacterium* sp.	99	JF730141.1
C24	*Variovorax* sp.	98	HQ730968.1
C25	*Paenibacillus* sp.	92	CP001656.1
C26	*Variovorax soli*	98	AB682436.1
C27	*Variovorax* sp.	95	HQ730968.1
C28	*Alcaligenes* sp.	97	JN166716.1
C29	*Cupriavidus metallidurans*	95	NC_007974
C30	*Rhizobium* sp.	78	DQ310799.2
C31	*Variovorax* sp.	96	EU741006.1
C32	*Stenotrophomonas maltophilia*	99	AB683956.1
C33	*Stenotrophomonas maltophilia*	96	HQ434490.1
C34	*Rhodospirillum centenum*	99	CP000613.2
C35	*Lysobacter* sp.	98	HE614876.1
C36	*Stenotrophomonas* sp.	99	EF219038.1
C37	*Lysobacter* sp.	96	DQ993327.1
C38	*Alcaligenes* sp.	97	JN166716.1
C39	*Alcaligenes* sp.	97	JN166716.1
C40	*Pseudomonas* sp.	99	JQ670732.1

Chapter 5. A method to isolate oxalotrophic bacteria able to disperse using fungal mycelia

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How important is the fungi-bacteria interactions in the OCP? How to assess the interaction between both microorganisms? What kind of interaction (synergist or antagonist) have been found related with the oxalotrophy and the OCP?

Chapter summary

This chapter presents the development of a new methodological approach to highlight the relevance of the interaction between two of the three biological players in the OCP: fungi and oxalotrophic bacteria. To assess the elements needed for an efficient OCP, it is necessary to understand the interactions between living organismas, and not only their presence in a shared ecological niche. The goal of this chapter is to show the role of filamentous fungi in the dispersal of bacteria able to consume and transform the oxalate sources around an oxalogenic tree. This interaction certainly increases the efficiency of the OCP occurring in tropical soils. To assess such complex ecological relationships from those organisms in soil, a new method to isolate natural couples of bacteria and fungi has been developed. This chapter will illustrate such approach with a case of study using soil samples from the Iroko tree, in Cameroon.

Abstract

A technique based on an inverted Petri dish system was developed for the growth and isolation of soil oxalotrophic bacteria able to disperse on fungal mycelia. The method is related to the "fungal highways" dispersion theory in which mycelial networks of fungi allow active movement of bacteria in soil. Quantification of this phenomenon showed that bacterial dispersal occurs preferentially in upper soil horizons. Eight bacteria and one fungal strain were isolated by this method. The oxalotrophic activity of the isolated bacteria was confirmed through calcium oxalate dissolution in solid selective medium. After separation of the bacteria-fungus couple, partial sequencing of the 16S and the ITS1 and ITS2 sequences of the ribosomal RNA genes were used for the identification of bacteria and the associated fungus. The isolated oxalotrophic bacteria included strains related to *Stenotrophomonas*, *Achromobacter*, *Lysobacter*, *Pseudomonas*, *Agrobacterium*, *Cohnella*, and *Variovorax*. The recovered fungus corresponded to *Trichoderma* sp. A test carried out to verify bacterial transport in an unsaturated medium showed that all the isolated bacteria were able to migrate on *Trichoderma* hyphae to re-colonize an oxalate-rich medium. The results highlight the importance of fungus-driven bacterial dispersal to understand the functional role of oxalotrophic bacteria and fungi in soils.

a. Introduction

Bacteria and fungi have probably co-existed since the development of deep soils on the Earth's surface. The emergence of fungi in terrestrial ecosystems must have had a strong impact on the evolution of bacteria, with soil bacteria living and evolving in a fungal world (Boer, et al., 2005). This co-existence has likely affected the bacterial niche development and fitness in positive and negative ways (Nazir, et al., 2010). An example of this is the association (loosely or tightly) of certain bacteria with mycorrhizal fungi, which apparently plays a role in mycorrhizal function (Bonfante & Anca, 2009). There is increasing evidence that bacteria-fungi interactions are widespread and may be crucial in ecosystem functioning (Johansson, et al., 2004, Boer, et al., 2005, Bonfante & Anca, 2009, Martin, et al., 2012).

The oxalate-carbonate pathway (OCP) is a biogeochemical pathway in which the roles of fungi and bacteria are instrumental (Verrecchia, et al., 2006, Cailleau, et al., 2011). In this pathway, the photosynthetic fixation of CO_2 by an oxalogenic tree leads to a flux of organic salts mainly in the form of calcium oxalate (Caox) into the soil. The biological oxidation of Caox and the modification of local soil pH produces the precipitation of calcium carbonate in unexpected geological settings (Cailleau, et al., 2004). Saprophytic fungi and oxalotrophic bacteria are the two microbial groups described as key players to modify the upcoming Caox flux (Verrecchia, et al., 2006).

The role of saprophytic fungi in the OCP is regarded as degrading organic matter and releasing plant-produced Caox from its embedding organic matrix. However, this needs to be revisited since fungal synthesis (Lapeyrie, 1988, Tuason & Arocena, 2009) and consumption (Guggiari, et al., 2011) of Caox has been shown previously. Moreover, a microcosm study investigating the importance of fungi and bacteria to modify soil pH demonstrated that only the simultaneous presence of oxalotrophic bacteria and fungi led to Caox oxidation in semi-natural conditions (Martin, et al., 2012). Nevertheless, the bases of the fungi-bacteria interactions occurring in the microcosm experiments could not be entirely unraveled.

One of the hypotheses to explain the role of fungi on the activity of oxalotrophic bacteria takes into account a phenomenon of bacterial dispersal in unsaturated media (such as a soil) that involves their active movement on fungal hyphae, which are thus used as so called "fungal highways" (Wong & Griffin, 1976, Wick, et al., 2007, Warmink & van Elsas, 2009). Fungal

highways are formed when hyphae cross air-filled pores connecting soil aggregates (Wösten, et al., 1999). Fungus-driven bacterial dispersal can help to explain the role played by fungi on the activity of oxalotrophic bacteria, in particular considering that Caox is a poorly bio-accessible substrate due to its low solubility and patchy distribution in soils (Graustein, et al., 1977, Tamer & Aragno, 1980, Messini & Favilli, 1990).

In order to demonstrate the existence of this dispersal mechanism in soils under the influence of the OCP, a method to recover oxalotrophic bacteria able to disperse on fungal hyphae has been developed. This method consisted of an inverted Petri dish that emulates a device proposed elsewhere for bioremediation studies (Furuno, et al., 2012). This method provides the conditions for the growth and recovery of autochthonous couples of fungi and oxalotrophic bacteria. The bacterial strains isolated were identified using the 16S rRNA gene, as well as the *frc* gene, a molecular marker used for the specific detection and identification of oxalotrophic bacteria (Khammar, et al., 2009). The fungus isolated was identified morphologically and by sequencing of the intergenic regions ITS1 and ITS2 (including the 5.8S rRNA gene) of the rRNA genes. Finally, a transport assay was carried out to confirm the dispersal of bacteria through an unsaturated medium. The isolation of these natural fungal-bacterial couples from soils represents an important step towards the comprehension of the role of fungi-bacteria interactions in soil microbial activity and functioning.

b. Material and Methods

i. Soil samples

Two soil profiles were studied in the region of Bertoua, Cameroon. Profile A (120 cm deep) was located near the trunk (17 cm) of a oxalogenic Iroko tree (*Milicia excelsa* (Welw.) C.C.Berg 1982). Profile C (140 cm deep) was sampled 15 m away from the tree, and assumed to be outside the influence of the oxalogenic tree (control soil). In each profile, 6 samples were collected at different depths. For profile A, the samples were collected at 2.5, 7, 13, 16.5, 45.5, and 55.5 cm. For profile C, the samples were collected at 2.3, 3.5, 6, 8, 27, and 92 cm. The samples were kept at 4°C.

ii. Isolation device and enrichment conditions

The isolation system consisted of Petri dishes used upside-down. One gram of a fresh soil sample was placed in the center of the Petri dish cover, leaving an air gap (around 7 mm) between the soil and the medium, which was placed on the top of the dish (Fig. 1A). The Petri dishes were incubated at 20±2°C for a week. The medium used was the Schlegel medium (Braissant, et al., 2002) supplemented with 4 $g.L^{-1}$ of calcium oxalate (Caox) monohydrate (Schlegel AB+Caox; Fluka 21201, Sigma Aldrich, Munich, Germany) as sole carbon source (Tamer & Aragno, 1980). The medium was poured in two layers. The first layer (ca. 20 mL) was prepared from solutions A and B (ratio 100:1) without the addition of the carbon source. The upper layer (ca. 5 mL) was prepared using the same solutions supplemented with Caox. The composition of solution A was (per L): $Na_2HPO_4.12H_2O$ 9 g; KH_2PO_4 1.5 g; NH_4Cl 1 g; $MgSO_4.7H_2O$ 0.2 g. One mL of trace-element solution DSM27 was added. The final pH of solution A was adjusted to 7.2. The composition of solution B was (in 250 mL): Fe(NH_4)citrate 0.125 g and $CaCl_2$.H2O 0.250 g. Solid medium was prepared by adding 1.6% agar (Biolife Italiana, Milan, Italy). Both solutions were sterilized in separate flasks due to immobilization of the KH_2PO_4 with Fe(NH_4)citrate. Sterilization was performed at 1 Pa., 120°C, during 20 min.

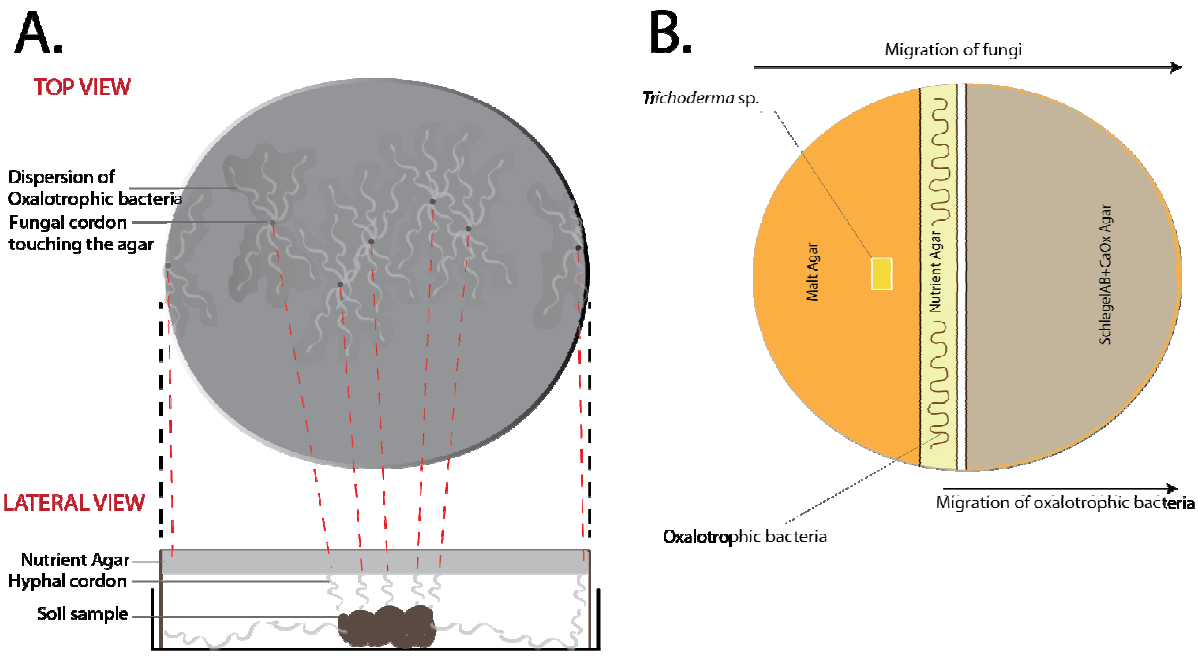


Fig. 1. A. Schematic representation of the inverted Petri dish device. The lateral view represents the relative position of the soil sample and the target medium (Schlegel AB with calcium oxalate –Schlegel AB-Caox). The top view shows the clearing halos in the medium after bacterial colonization associated to the formation of a hyphae link between the soil and the target medium. **B**. Schematic representation of the experimental device for the confirmation test of bacterial dispersion along fungal hyphae. The inoculation in this assay was performed using Malt agar to favor fungal growth (left), nutrient agar to benefit bacterial growth (middle), and Schlegel AB-Caox as target medium (right) separated by a 2 mm air space between the target medium and the nutrient agar.

The number of oxalotrophic bacteria transported to the Schlegel AB+Caox was estimated by colony forming unit (CFU) counting using clear halos around the bacterial colonies as indicative of Caox dissolution (Tamer & Aragno, 1980). In all the cases, the contact between fungal hyphae and the medium where oxalotrophic bacterial colonies were developed, as well as the absence of micro invertebrates that could contribute to bacterial dispersal, was verified visually with the help of a binocular. All the colonies observed were connected to hyphae and the typical dispersal pattern associated to micro invertebrates (strain of successive colonies) was never observed.

iii. Isolation of oxalotrophic bacteria and fungi

Bacterial colonies showing dissolution halos were selected as oxalotrophic and isolated in fresh Schlegel AB+Caox medium. Petri dishes were incubated at 20±2°C during a week. The fungus associated with oxalotrophic bacteria was recovered by taking a portion of fungal filaments that

have made contact with the solid medium and allowed the development of oxalotrophic bacteria. The fungal filament was incubated in 12 g L^{-1} malt agar 1.5% (w/v; Biolife, Milan, Italy). There was no addition of antibiotics to control bacterial growth. The selection of fungal filaments was made regarding the morphological differences of the associated bacterial colonies. In this specific case, all the bacterial colonies were associated with the same fungus (according to the morphological description) and therefore, only one fungal partner was isolated. However, it is important to mention that the short incubation period (one week) could have favored fast-growing fungi and the incubation system does not allow the recovery of mycorrhizal fungi. The oxalotrophic bacteria and the associated fungus were preserved in glycerol and physiological solution 1:1 (v/v) and stored at -80°C.

iv. Characterization of bacterial strains and the associated fungus

Pure bacterial strains were grown in solid Schlegel AB+Caox medium prior to DNA extractions using the Analytik Jena InnuPrep Bacteria DNA extraction kit (Analytik Jena AG, Jena, Germany). Modifications for Gram-positive bacteria were applied to the provider's protocol. A sonication pre-step was performed on the biomass at 60 mA during 7 s in order to dissolve flocks formed by filamentous isolates. Digestion with lysozyme was increased from 30 to 45 min at 37°C. The incubation with Lysis solution was increased from 10 to 20 min. at 50°C. DNA extracts were quantified using a Nanodrop® spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, USA). DNA concentration ranged from 21 to 64 ng μL^{-1}. The DNA extracts were conserved at -20°C in 100 µL of the elution buffer provided with the extraction kit. A few hyphae from the pure fungal culture were used directly for PCR amplification.

PCR amplification of a partial fragment of the 16S rRNA gene with the primers Eub9_27 forward (5'-GAGTTTGATCCTGGCTCAG-3'), and Eub1542 reverse (5'-AGAAAGGAGGTGATCCAGCC-3') (Liesack, et al., 1991) were carried out for the identification of the bacterial strains. For fungal identification, a set of primers for the amplification of the ribosomal ITS sequences 1 and 2 including the 5.8S rRNA gene was used (White, et al., 1990). This region enables a precise identification of fungi between and within genera (Buchan, et al., 2002). The PCR reaction mixtures used for both bacterial and fungal DNA amplification contained (in 50 µL of final volume): 1X Standard Buffer (Biolabs, New England, Ipswich, MA, USA) with 1.5 mM $MgCl_2$, 0.2 mM dNTPs mix (Promega AG, Dübendorf, Switzerland), 0.2 µM of each primer (for bacterial or fungal amplification), and 1 U of Standard DNA Taq polymerase (Biolabs, New England, Ipswich, MA, USA). A total of 2 µL

of diluted DNA (ca. 2 - 2.6 μg μL_{-1} of DNA) was added as a template. The initial denaturation was carried out at 94°C for 4 min 30 s, followed by 30 cycles consisting of denaturation at the same temperature for 30 s, annealing at 60°C for 30 s, and extension at 68°C for 1 min 30 s. A final extension was performed at 68°C for 10 min. All PCR reactions were carried out in a thermocycler Sensoquest Labcycler (Witec A.G., Göttingen, Germany). PCR products were visualized by gel electrophoresis using agarose gels 1% (w/v) run 30 min at 90 V and 60 mA in a horizontal electrophoresis chamber (VWR, Fontenay-sous-Bois, France) with 200 mL of TBE buffer 0.5 X.

The purification of PCR products was carried out using 96-well filtering plates from Millipore (Millipore AG, Zug, Switzerland). The PCR product was premixed with an equal volume of TE buffer 1X and filtered during 15 min, adding 50 μL of nanopure sterile water afterwards. The amplicons were quantified using a Nanodrop® spectrophotometer, and sent for Sanger sequencing to GATC-Biotech AG (Konstanz, Germany). DNA concentration from the amplicons sent for sequencing ranged from 24 to 82 ng μL^{-1}. The search for similarity against sequences from the gene 16S rRNA or the ITS was performed using BLASTn (Altschul, et al., 1997) comparing the query sequence with the non-redundant GenBank database at the National Center for Biotechnology Information (NCBI).

For oxalotrophic bacteria, in addition to the 16S rRNA gene, a fragment of 155 bp of the *frc* gene was amplified with the primers frc-171-f (5'-CTSTAYTTCACSATGCTSAAC-3') and frc306-r (5'–GDSAAGCCCATVCGRTC-3') (Khammar, et al., 2009). The PCR mix contained the same products described for the amplification of the 16S rRNA gene except that the primers for the frc gene amplification (frc-171-f and frc306-r) were used at 1.25 μM each. The initial denaturation step was performed at 94°C for 5 min. The reaction mixtures were subjected to 35 amplification cycles. Cycles consisted of denaturation at 94°C for 30 s, primer annealing at 56°C for 1 min 30 s, and extension at 68°C for 45 s, with a final extension at 68°C for 10 min. The PCR was carried out in a thermocycler Bio-Rad MJ Mini PTC-1148 (BioRad, A.G. Munich, Germany).

The 16S rRNA gene (bacteria) and 18S-26S rRNA intergenic region (fungi) sequences from the isolates were deposited in GenBank under accession numbers to be assigned (Sequences submitted to GenBank on July 2th 2013).

v. Transport confirmation test

In order to verify the relevance of fungus-driven bacterial dispersal, a motility test was conducted for all the isolated bacterial strains with the associated fungus *Trichoderma* sp. This test consisted of a modified Petri dish in which heterogeneity was generated by changing the substrate media and including an air gap between the donor and target media (Fig. 1B). Three solid media were selected for this test, taking into account the ability to grow for each one of the microorganisms assessed. A fragment of 3.5 cm (width at the center) from malt agar (MA; Biolife, Milan, Italy) was placed in an empty Petri dish. This medium was the donor medium for fungal growth. A fragment of 1 cm (width at the center) from nutrient agar (NA; Biolife, Milan, Italy) was placed next to it in close contact with the fungal donor medium as medium for bacterial growth. Finally, a fragment of 4.5 cm (width at the center) from Schlegel AB+Caox was placed in the other side of the Petri dish. This medium was the target medium, specific for oxalotrophic growth. A gap of 2 mm between the NA and the target medium was included to ensure fungus-driven bacterial dispersal as the sole possibility to find oxalotrophic bacteria in the target medium. A 24 h old fungal strain was inoculated under sterile conditions in the center of the MA fragment. Oxalotrophic bacteria were inoculated as a lawn in zig-zag around the NA fragment. Controls were consisted in Petri dishes inoculated with oxalotrophic bacteria without the fungal partner, to be sure that bacteria cannot reach the target medium by themselves. The plates were incubated for 2 weeks at room temperature. After clear colonization by the fungal mycelium of the target medium, the presence of bacteria (which were not easily detectable by direct observation) was confirmed by PCR using the 16S rRNA and frc genes. In addition, a similar experiment using only NA (donor) and Schlegel AB+Caox (target) media was carried to verify the specificity of the interaction. In this case, the fungus was replaced by glass fibers (Glass wool 8799C Cole-Parmer IL, USA) with a similar diameter to hyphae (± 8 μm) and bacteria colonization was verified as described above. Growth was monitored during 9 days of incubation at room temperature. The plates were observed microscopically (DigiMicroscope USB Reflecta GmbH, Rottenburg, Germany) and a Gram staining was carried out to observe bacteria associated to the glass fibers using a Leica DMR Trinocular Industrial Microscope, adapted with a digital camera DXM 1200 Nikon (Leica Microsystems AG, Heerbrugg, Switzerland).

c. Results and discussion

In the present study, the use of a simple experimental approach consisting of the physical separation of soil material and a target selective medium for oxalotrophic bacteria, allowed an initial observation and quantification of the phenomenon of fungus-driven oxalotrophic bacterial dispersal in soils. Fungus-driven bacterial dispersal was observed at six depths (from 0 down to 56 cm) of the profile positively influenced by a flux of Caox from the oxalogenic Iroko tree (profile A; Fig. 2A). Eight to 60 colony-forming units (CFU) per g of soil were observed (Fig. 2B). In comparison, only a single colony was obtained for the control soil (Fig. 2B), even though fungal growth was observed in the upper 20 cm (data not shown). Quantification by conventional plating showed the presence of oxalotrophic bacteria of the order of 10^7-10^8 CFU per g of soil for profile A and 10^7 CFU per g of soil in the upper 5 cm of profile C, which is expected from this generalistic bacterial guild. Therefore, it is surprising to observe such a restricted effect on the number of bacteria dispersing using fungal mycelia in the control soil, and particularly a marked rhizospheric influence of the oxalogenic tree. The positive influence of oxalogenic trees on oxalotrophic bacteria has been observed in the past (Braissant, et al., 2004, Cailleau, et al., 2004). However, these new results suggest that this influence extends to the interaction between fungi and oxalotrophic bacteria. A previous study on the impact of white-rot fungi on the composition and abundance of bacteria colonizing decaying wood in a forest soil showed the positive influence of oxalogenic fungi on colonization by oxalotrophic bacteria (Folman, et al., 2008). The results presented here are yet another indication of the potential ecological role of the interaction between bacteria, fungi, and flux of Caox in soils.

One fungal strain and eight bacterial strains (Table 1) were isolated in this study. The morphological characteristics of the fungal colonies and the microscopic structures of the conidiophores correspond to *Trichoderma* sp. (Hoog & Guarro, 1996). This was confirmed by the sequencing of the ITS1 and ITS2 regions of the ribosomal RNA gene, used for fungal identification (Buchan, et al., 2002). On the other hand, seven of the bacterial strains were affiliated to different genera of the class Proteobacteria, including strains related to *Stenotrophomonas*, *Achromobacter*, *Lysobacter*, *Pseudomonas*, *Agrobacterium*, and *Variovorax*. From those, the genera *Pseudomonas*, *Agrobacterium*, and *Variovorax* have been reported as oxalotrophic in other studies (Sahin, 2003). The strain *Cohnella phaseoli* WCA6 (belonging to the Class *Bacilli*), corresponded to a genus that has not been previously reported as oxalotrophic, nor has been recovered by traditional plating. Several studies have listed bacterial genera in the mycosphere in

the past (Linderman, 1988, Linderman, 1991, Johansson, et al., 2004, Folman, et al., 2008, Warmink & van Elsas, 2009, Scheublin, et al., 2010). The detection of these bacteria has been achieved e.g. by using artificial compartmentalized systems to "capture" bacteria colonizing hyphae (Scheublin, et al., 2010), or in experiments with soil collected near fungal fructifications (Warmink & van Elsas, 2009). An emerging picture from those studies is that some bacterial groups are prone to associate with fungi. These include strains belonging to *Pseudomonas*, *Agrobacterium* and *Burkholderia*, which were also observed here. However, the present study provides evidence for a greater diversity.

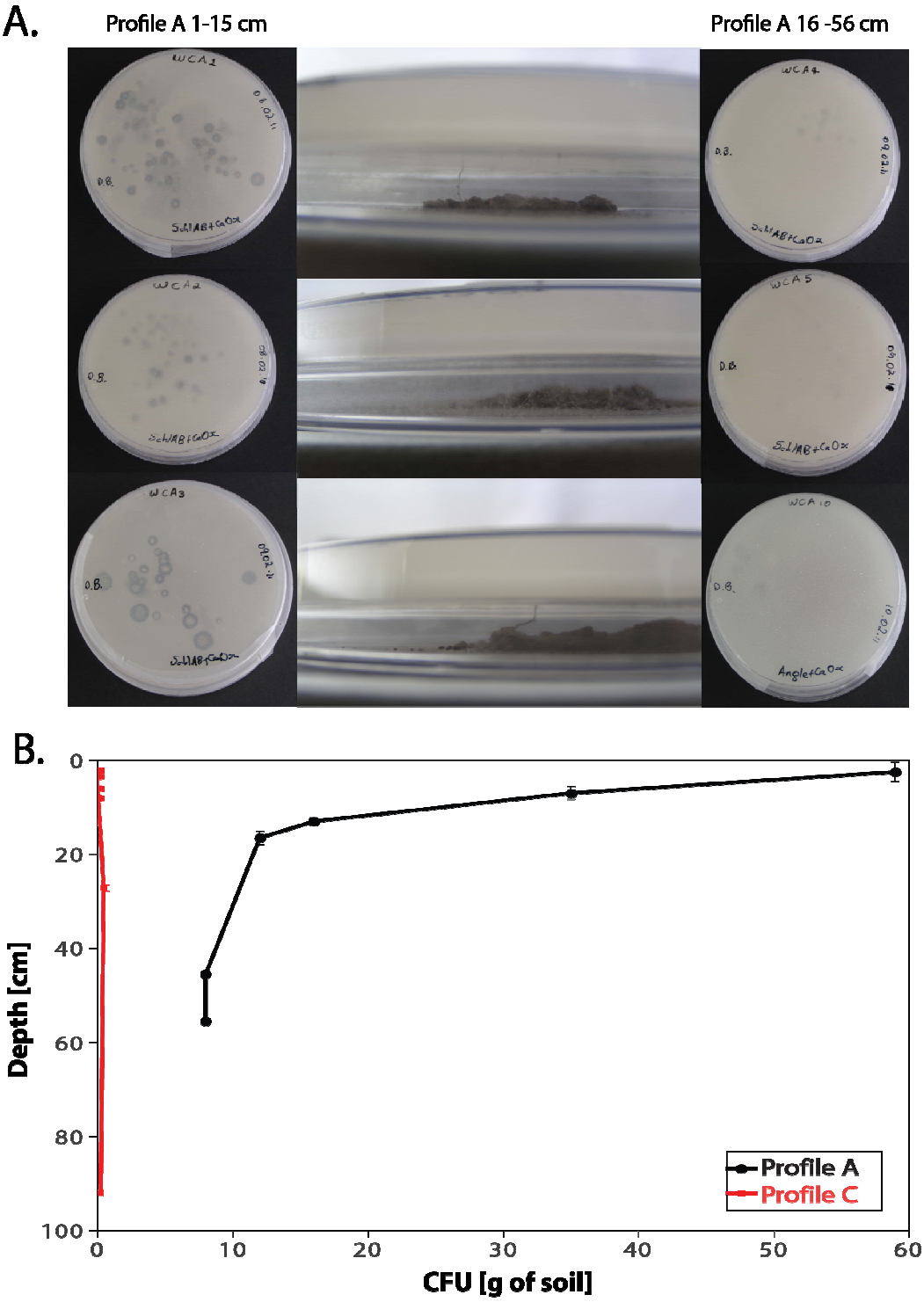


Fig. 2. Quantification of fungus-driven bacterial dispersal associated to the flux of calcium oxalate in soil. **A**. Images showing the growth of oxalotrophic bacteria isolated using the inverted Petri dish with Schlegel AB-Caox as target medium. Images from the middle part show the physical contact by fungal hypha filaments (indicated with white arrows) growing from the soil and up, reaching the agar in the upper part of the Petri dish. **B**. Colony-forming unit (CFU) counting in two soil profiles performed with soil samples from Cameroon. Profile A (solid line) corresponds to the soil sampled aside the oxalogenic tree *Milicia excelsa*. Profile C (dashed line) corresponds to soil collected distantly from the tree trunk.

All the strains were confirmed as oxalotrophs by re-plating on Schlegel AB-Caox, however not all of them were positive for the amplification of the *frc* gene (Table 1), a molecular marker used to identify oxalotrophs (Khammar, et al., 2009). This gene is critical as it encodes for the formyl coenzyme A transferase enzyme, which is essential for oxalate catabolism (Svedruzic, et al., 2005). A bias in amplification could be the result of sequence variations and problems in primer coverage. However, it is worth mentioning that, while growth in Schlegel medium is evident for the two frc-negative strains, the clearing halo for Caox is not (Fig. 3B and Fig. 3D). Consequently, the mechanism of oxalate degradation in *Achromobacter* sp. WCA2 and *Pseudomonas* sp. WCA4 needs to be investigated further.

Table 1. Description of eight bacterial strains isolated and characterized using the inverted Petri dish assay and the "fungal highway" concept for dispersion of oxalotrophic bacteria. Strains were identified by sequencing the 16S rRNA gene. After migration in fungal hyphae DNA was extracted and the presence of bacteria in the target medium was confirmed by amplification of the 16S rRNA and *frc* genes. The frc amplification confirmed the results obtained with the pure cultures since *Achromobacter* sp. WCA2 and *Pseudomonas* sp. WCA4 did not amplify the *frc* gene.

						After migration	
Strain	**First hit strain in BLAST**	**Gram**	**Flagella[a]**	**Class**	***frc* gene**	**16S rRNA**	***frc* gene**
WCA1	*Stenotrophomonas* sp.	+	Twiching	γ-Proteobacteria	+	+	+
WCA2	*Achromobacter* sp.	-	+	ß-Proteobacteria	-	+	-
WCA3	*Lysobacter gummosus*	-	Gliding	γ-Proteobacteria	+	+	+
WCA4	*Pseudomonas* sp.	-	+	γ-Proteobacteria	-	+	-
WCA5	*Agrobacterium* sp.	-	+	α-Proteobacteria	+	+	+
WCA6	*Cohnella phaseoli*	+	+	Firmicutes	+	+	+
WCA7	*Variovorax soli*	-	+	ß-Proteobacteria	+	+	+
WCA8	*Variovorax* sp.	-	+	ß-Proteobacteria	+	+	+

[a] The presence or absence of flagella was described according previous studies (Stanier, et al., 1966, Christensen & Cook, 1978, Merritt, et al., 2007, García-Fraile, et al., 2008, Jamieson, et al., 2009)

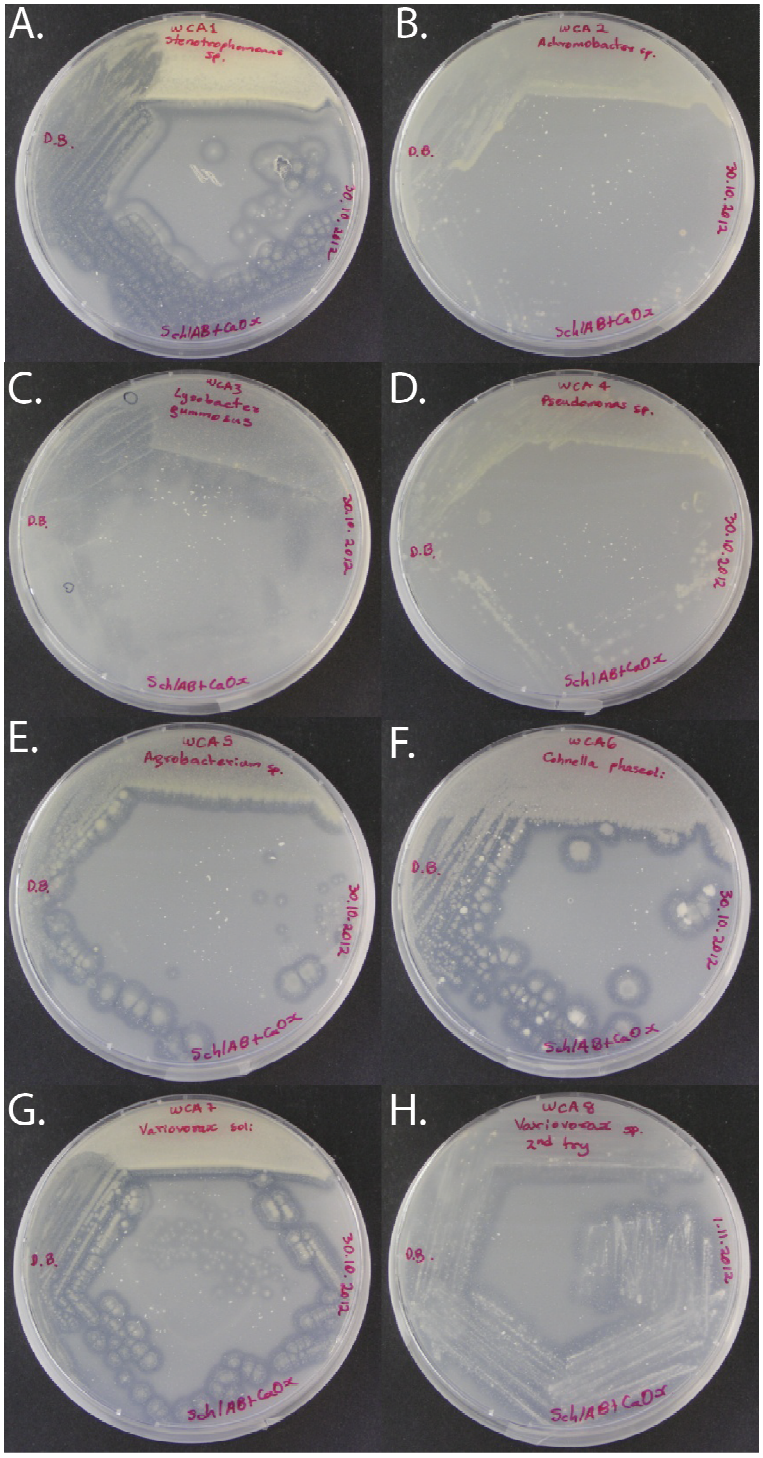


Fig. 3. Degradation halos observed after growth of the bacterial strains isolated by the inverted Petri dish method (see Table 1). The medium corresponds to Schlegel AB medium with calcium oxalate as sole carbon source. The degradation halo corresponds to the dissolution of calcium oxalate that gives a milky aspect to the medium. **A**. *Stenotrophomonas* sp. WCA1; **B**. *Achromobacter* sp. WCA2; **C**. *Lysobacter gummosus* WCA3; **D**. *Pseudomonas* sp. WCA4; **E**. *Agrobacterium* sp. WCA5; **F**. *Cohnella phaseoli* WCA6; **G**. *Variovorax soli* WCA7; **H**. *Variovorax* sp. WCA8.

On the other hand, one of the features apparently shared by bacteria able to disperse on fungal mycelia includes flagellar motility (Kohlmeier, et al., 2005). A literature survey (Table 1) shows that six out of the eight genera possess flagella and a motility mode (swimming or swarming) associated to it (García-Fraile, et al., 2008, Jamieson, et al., 2009). For the two other genera, twitching (*Stenotrophomonas* sp. WCA1) and gliding (*Lysobacter* sp. WCA3) motility has been reported (Aslam, et al., 2009, Ryan, et al., 2009). Thus, mobility appears to be a common characteristic for bacteria dispersing on fungal mycelia. However, an issue that needs to be considered is how relevant is fungus-driven bacterial dispersal versus self-dispersal in soil. In studies under controlled conditions, it has been postulated that flagellated bacteria would be expected to swim or to swarm during saturation events that are often short and sporadic in soils (Dechesne, et al., 2010). In contrast, mycelia are highly abundant in most soils with estimates of a mycelia network that represents up to 1000 m per g of soil (Kohlmeier, et al., 2005). Hence, naturally available fungal networks could offer a permanent option for dispersal of flagellated bacteria (oxalatrophic or not).

In order to verify the migration capabilities of the bacterial strains on the isolated fungus, a migration assay was performed. Fungal growth in the air-filled space between the donor (MA and NA) and the target (Schlegel AB-Caox) media was observed after only a few days of incubation (Fig. 4A). Bacterial dispersal to the target medium was not clear to the naked eye and therefore a confirmation assay by PCR was devised. Indeed, this assay showed that all the strains migrated using the fungal hyphae to the target medium (Table 1), confirming that this is a potential dispersal mechanism for those bacterial strains. Dispersal was also confirmed in test using glass fibers connecting nutrient agar and Schlegel AB with Caox for all the strains (Supplementary Figure 1), suggesting that oxalotrophic bacteria clearly take advantage of the physical dispersal network offered in soil by fungal hyphae.

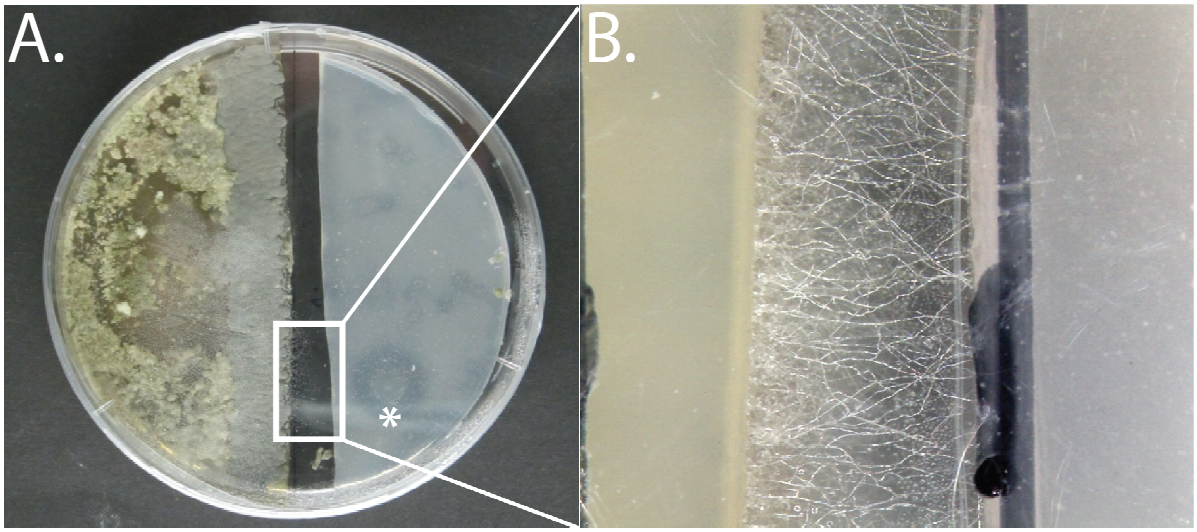
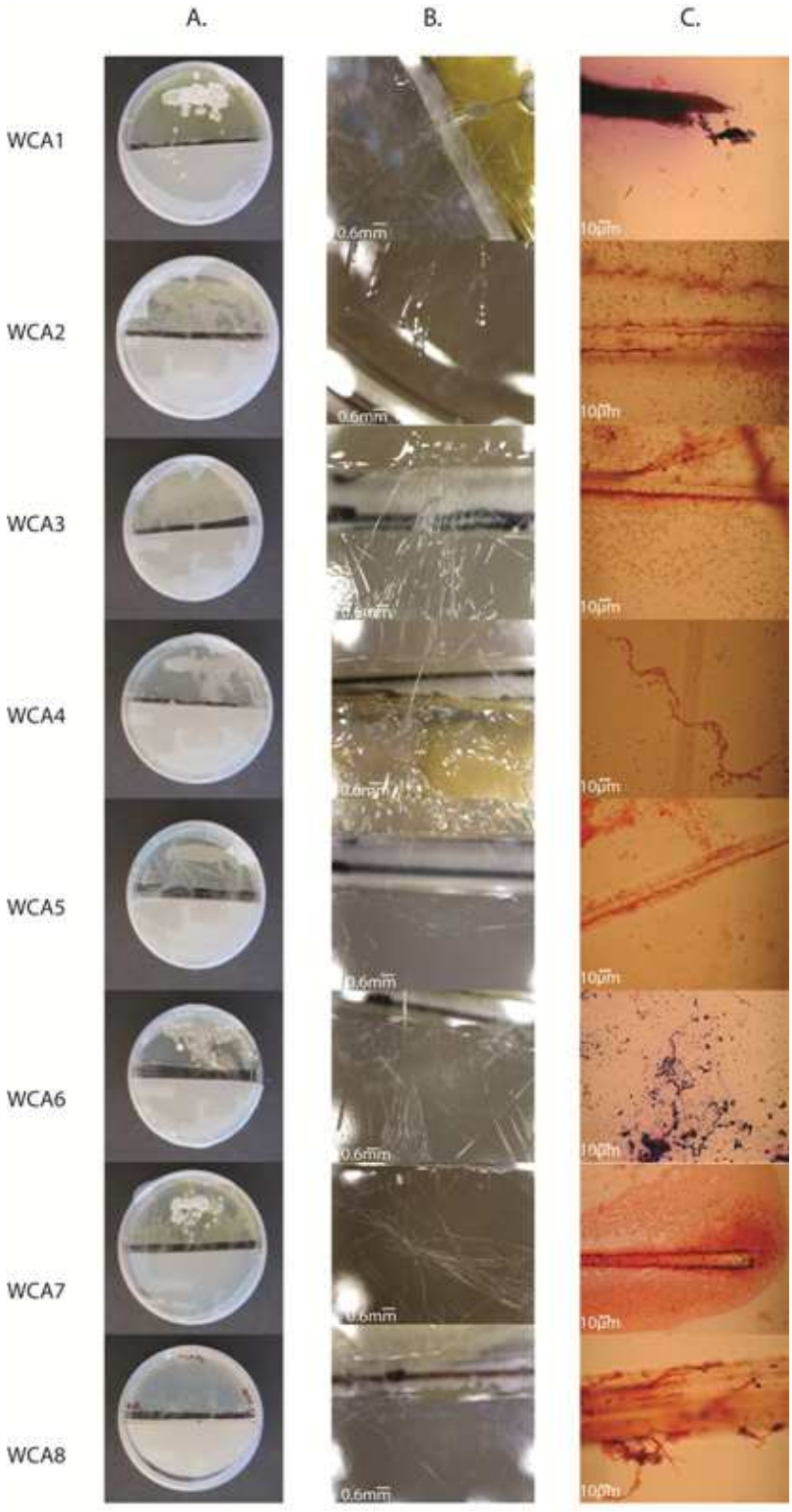


Fig. 4. Test results of bacterial dispersion along fungal hyphae. **A**. The interaction with *Trichoderma* sp. allowed the tested oxalotrophic bacteria (here *Stenotrophomonas* sp. WCA1) to access the target Schlegel AB-CAox medium. The dispersion of oxalotrophic bacteria is due to fungal highways formation through the air gap, and is confirmed by degradation halos of Caox in the target medium (indicated by an asterisk). **B**. Close-up at the fungal growth through the air-filled gap observed after 5 days of incubation (scale bar = 0.6 mm).



Supplementary Fig. 1. Test results of bacterial dispersion along fungal hyphae using glass fibers that simulate hyphal filaments. A. Petri dishes with bacterial growth after 9 days of incubation. B. Stereoscopical microscopy showing bacteria dispersed through the target Schlegel medium with Caox. C. Gram staining showing tested oxalotrophic bacteria attached to the glass fibers, collected at the interface between nutrient agar and target Schlegel media.

In summary, this study demonstrates the importance of bacteria-fungi interactions in the oxalate carbonate-pathway. The method of the inverted Petri dish is an interesting approach to isolate and study the ecological implications of fungal-bacterial couples found in terrestrial environments related with the OCP. Although the experimental system is simple, and can be further improved, the results obtained here raise important questions regarding the implications of fungi-bacteria interactions in such soils. For example, it would be important to establish the existence of cooperation (synergy) or competition (antagonism) between the couples of microorganisms involved in the pathway. In the case shown in this study, the interaction favors bacterial dispersal to access Caox as carbon and energy source, which could be significant in soil found near an oxalogenic tree. Nonetheless, in other cases, oxalotrophic bacteria could protect plants against phytopathogenic fungi, as it has been demonstrated in previous studies with rhizospheric oxalotrophic bacteria associated to *Arabidopsis thaliana*, playing the role of antagonist against the phytopathogen *Botrytis cinerea* (Schoonbeek, et al., 2007). In addition, new pathways could be discovered related to the metabolism of oxalate favored by the interaction bacteria-fungi. For example, enzymes potentially involved in the production of energy from oxalate have been described recently in fungi (Watanabe, et al., 2005), and this can have an effect on the associated bacteria. All these elements need to be considered in the future to understand the interaction between fungi and bacteria in soils, as well as effects of these interactions in soil functioning.

d. Acknowledgements

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Chapter 6. Global discussion: Comparison of the oxalate-carbonate pathway between continents and the contribution of this work to the knowledge of the OCP

Daniel Bravo

How diverse oxalotrophic bacteria are?
What are the perspectives of this work?

Chapter summary

This chapter presents a comparison between oxalotrophic bacteria found in the three OCP systems assessed. This comparison should generate a new perspective about diversity and metabolism of oxalotrophic bacteria. The goal of this chapter is to point out that such a comparison of oxalotrophic bacteria in terms of diversity and metabolism is an image of the efficiency of the OCP occurring in Bolivia, India and Cameroon. Finally, a brief comment about the contribution of this work to the general knowledge in the OCP and the perspectives and application of the results are presented.

This chapter discusses the main results presented in the previous chapters. The aim of this thesis was to assess the diversity and metabolism of oxalotrophic bacteria in tropical habitats. This goal was developed through four main questions (Figure 1). Those questions allow a better understanding of the role of oxalotrophic bacteria in the oxalate-carbonate pathway from the tropical systems assessed.

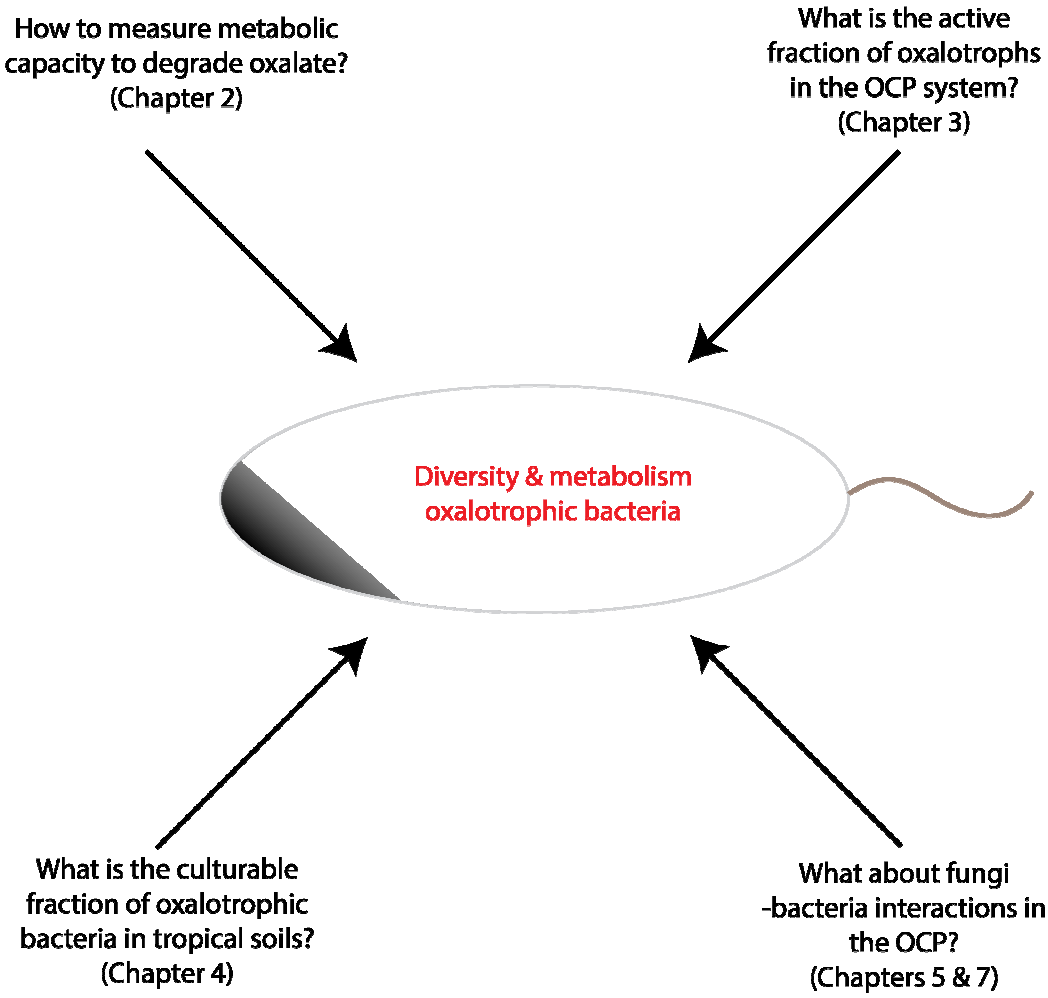

Figure 1. Main questions that have been developed during my doctoral thesis. Each venue has elucidated a new aspect of the overall vision on diversity and metabolism of oxalotrophs in the OCP from tropical forests.

To assess the diversity and metabolism of oxalotrophs in tropical soils was necessary to develop two approaches that implied thermodynamics of bacterial growth and molecular biology of replicating DNA from oxalotrophic bacteria. Each approach was considered independently in chapters 2 and 3 and the main contributions to the studied subjects are highlighted in the next

paragraphs. Furthermore, the culturable fraction of oxalotrophs was considered in chapter 4. The application of such isolation and characterization of strains is discussed at the end of this chapter.

Finally, an important issue in the study of the oxalate-carbonate pathway that has not been considered before is the participation of the biological players and their interactions. This is discussed in chapters 5 and 7. In these two chapters the influence of fungi-bacteria interactions and the shift on local pH (which favors the precipitation of secondary calcite) are demonstrated. Nevertheless, many questions still remain about the biological interaction and its contribution in the storage of carbon from atmospheric CO_2 into $CaCO_3$ though the OCP. Some of those questions will be discussed in this section.

As it was explained in chapter 1, the OCP is a biogeochemical pathway involving biological terrestrial organisms and geological processes. It was the purpose of this work to contribute in the knowledge of a specific group of participants on the pathway, with positive implications on the global cycle. Nevertheless some questions that remained unanswered and should be taken into account for further studies include the role of mycorrhizal interactions in the pathway, as well as the factors determining the biogeography of the oxalotrophic bacteria found in tropical soils. These elements could have an impact on the applications of the OCP in agricultural forestry, soil management, and CO_2 stockage strategies using the obtain results. Likewise, a deep analysis of biotic and abiotic factors to compare the three sites studied will be taken into account in a manuscript in preparation (Caillieau, Bravo et al. 2013), but none of the former are part of this global discussion.

Highlights of the thesis

Following the outline of the thesis, the chapter one has shown the current knowledge about the OCP, oxalotrophic bacteria and diversity and metabolism of this group in tropical habitats. It was highlighted the fact that our knowledge in those particular environments was incipient specially dealing with the metabolic capability and diversity of non-culturable oxalotrophs in tropical forests.

Chapter 2 has introduced the isothermal microcalorimetry as a new non-destructive approach to assess metabolism of oxalotrophic bacteria *in situ*, or *in vitro*. Through that chapter, it was shown that bacteria could be classified as fast or slow growing oxalotrophs in function of the consumption rate of oxalate as sole carbon and/or energy source. It has been shown as well that

measuring oxalate consumption rate is possible in soil, to assess the whole activity of oxalotrophic community present in those soils. The addition of Caox estimulated positively the oxalotrophic activity with a heat production (in Joules) comparable to that heat obtained when an oxalotrophic model strain has been grown in oxalate. Interestingly, two peaks of activity have been recorded in soil. The first correspond most likely to direct oxalate consumption (fast growing oxalotrophic bacteria) and the second could represent the use of a less accessible source of oxalate (slow-growing oxalotrophic bacteria). Therefore, the dispersal of bacteria to access oxalate in soil was a remaining question that was re-examined in chapters 5 and 7.

Chapter 3 has pointed out the importance of molecular tools to study non-culturable bacteria and specifically the metabolically active fraction of the oxalotrophic community in soils. To determine that fraction, a new approach was developed using the BrdU-labeling *frc* assay and the DGGE profile. With this study it has been shown that the input of Caox into soil is one of the limiting factors for bacterial oxalotrophic activity; the soil pH shift towards alkalinity as an effect of oxalotrophic activity, and interestingly, those different Caox concentrations are not driving forces that modify drastically the composition of the oxalotrophic communities. The results had pointed out that an important fraction of active bacteria corresponds to non-phylogenetic related rizhospheric oxalotrophs. In addition, the presence of two populations that appear in the active fraction regardless the concentration of Caox amended was highlighted. Those were *Kribbella flavida* and *Streptomyces* sp., both Actinobacteria. Therefore, linking those results with the method developed, new insights in the knowledge of oxalotrophic diversity of active non-culturable actinobacteria, which otherwise could be easily underestimated by plating methods, was achieved.

Chapter 4 gives new insigths into the diversity of oxalotrophs isolated from Bolivian, Indian, and Cameroon OCP systems. The chapter shows an important effort to prepare a collection of oxalotrophic bacteria from tropical soils. The collection was set-up regarding physiological features such as the degradation halos of Caox observed around colonies growing in the surface of selective medium. Ten strains were characterized using IMC, HPLC chromatograms of oxalate consumption, and biochemical tests to know their metabolic capabilities to consume other carbon sources. Moreover, it has been highlighted the ecological role of strains involved in the OCP with high oxalate consumption rates, such as *Variovorax soli* C18, *Lysobacter* sp. A8, *Agrobacterium* sp. C23, and *Streptomyces achromogenes* A9 (pointed out by it high biomass production).

Chapter 5 revise the question remaining at the end of chapter 2 about the dispersal of oxalotrophic bacteria, pointing out fungi as instrument of transport for oxalotrophic bacteria to access to oxalate in soils. A new method was developed to isolate autochthonous couples of fungi and oxalotrophic bacteria. A simple device consisting of inverted Petri dishes has shown to be a powerful tool to analyze both fungi and bacteria, related with the oxalate reservoirs in soil across a vertical profile. This new method is relevant for the comprehension of oxalate release and dispersion, which have a direct effect over oxalotrophic communities' dispersion and advantages to use a transport system such as fungal hyphae. This chapter let open the door to many ecological questions that should be revised in further studies. For instance, what are the primarily ecological relationships between fungi and bacteria around oxalate? Is there a synergism or an antagonism around the access to oxalate? Could be possible to establish a pattern of distribution or dispersion (reservoirs-like) of oxalate at the end of the fungal highways? (Round about-like micro-spaces, in which oxalate is abundantly released/displaced probably near to the root systems of the tree).

Chapter 7 extent our knowledge about the fungi-bacteria interaction at a microcosm scale. This chapter considers that changes in the pH are a consequence of oxalotrophy and enhanced due to fungi-bacteria interactions. The presence of both biological players is highlighted for an efficient OCP. This study has shown that changes in the composition of total bacteria occur when oxalate, fungi and bacteria are amended. In addition, the method of inverted Petri dish was evaluated with oxalotrophic model strains (*Panderaea* sp., *Oxalicibacterium flavum*, *Ancylobacter polymorphus, Streptomyces violaceoruber, Cupriavidus necator*, and *Variovorax paradoxus*), three non-oxalotrophic model bacteria (*Bacillus subtilis*, *Escherichia coli*, and *Pseudomonas aeruginosa*), and three environmental fungi (*Pycnoporus cinnabarinus*, *Trametes versicolor*, and *Polyporus ciliates*). The results suggested that *Pandoraea* sp., *A. polymorphus*, and *C. necator* were able to migrate from an unfavorable to a favorable substrate by crossing the liquid-air gap with the aid of fungal highways. The beneficial effect of fungi on bacterial development was highlighted. It was hypothesized that exhudation of certain compounds by fungal hyphae would favor the attachment of fungi-associated bacteria, therefore, playing a protective role with oxalate-oxidizing bacteria.

The contribution of this work to our current knowledge in the OCP.

In figure 1 was shown four main questions that were developed in this study. The first two questions were related with metabolic capabilities and identification of active oxalotrophs in their environment. The development of IMC and BrdU-*frc* assays reflect the interest to understand both aspects. Indeed, those techniques have an important impact over our understanding in oxalotrophy found so far.

Isothermal microcalorimetry has been applied to understand the metabolism with soil samples and oxalotrophs isolated from Bolivia, India, and Cameroon OCP systems. The technique has been used to determine which oxalate source in soil could be the preferred by oxalotrophic bacteria, showing that effectively Caox and Kox were the best oxidized substrates due to oxalotrophic metabolism in environmental strains and soil samples, indicating that IMC could be easily used as a diagnostic criterium to select sources in specific metabolisms such as in this case oxalotrophy. Currently, IMC is used in the study of fungi-bacteria interactions in other thesis related with the OCP in semiarid environments. In addition, both IMC and BrdU-*frc* techniques allow widening the collaboration research network in the study of this functional group.

BrdU-frc DGGE profile has increased our knowledge about the active fraction of the oxalotrophic community at a microcosm scale using the OCP system from Cameroon. This is the first time that active oxalotrophic non-culturable bacteria are identified using labeling as a proxy of the populations in an OCP system in tropical habitats. As a consequence, the phylotype *Kribbella flavida* was reported for the first time as active oxalotrophic non-culturable bacteria.

The new methods developed here were able to improve studies *in vitro* but also *in situ* conditions. This is a big contribution in comparison with the traditional culturing work carried out in previous studies with oxalotrophic bacteria (Sahin*, et al.*, 2002, Tamer*, et al.*, 2002, Sahin, 2003, Braissant*, et al.*, 2004).

Furthermore, isolation of oxalotrophic bacteria was successfully achieved in this thesis. Ninety-four strains were isolated from Bolivia, India, and Cameroon. Ten selected strains were completely characterized by IMC, HPLC quantification of oxalate consumed, kinetic growth curves, and biochemical tests. Moreover, 12 new genera were reported as oxalotrophic strains from tropical habitats for the first time (Table 1).

Table 1. Literature about diversity of oxalotrophic bacterial diversity at the date of this publication. In red genera characterized in the current research.

Oxalotrophic strain	**Ref.**
Acinetobacter sp. *Agrobacterium sp.* *Alcaligenes* *Ammoniphilus oxalaticus*	(Sahin*, et al.*, 2008)
Ancylobacter sp. *Azospirillum brasilense*	(Sahin, 2003)
Azorhizobium oxalatiphilum *Bacillus licheniformis* *Beijerinckia indica* *Burkholderia sp.* *Carbophilus carboxydus*	(Lang*, et al.*, 2013)
Cupriavidus oxalatcus *Desulfovibrio vulgaris* *Eggerthella lenta* *Enterococcus faecalis* *Hyphomicrobium vulgare*	(Sahin*, et al.*, 2002)
Lactobacillus spp. *Mesorhizobium sp.*	(Lewanika*, et al.*, 2007)
Methylobacterium extorquens *Methylobacterium spp.* *Moorella thermacetica* *Mycobacterium spp.*	(Tamer*, et al.*, 2002) (Trinchant & Rigaud, 1996)
Nocardia sp. *Oligrotropha carboxydovorans* *Oxalicibacterium flavum* *Oxalicibacterium flavum*	(Knutson*, et al.*, 1980)
Oxilicbacterium spp. *Oxalobacter formigenes* *Oxalophagus oxalicus*	(Sidhu*, et al.*, 1997, Sahin*, et al.*, 2010)
Pandoraea oxalativorans *Paracoccus alcaliphilus* *Providencia sp.* *Pseudomonas spp.* *Ralstonia eutropha*	(Sahin*, et al.*, 2010)
Rhizobium leguminosarum *Saccharothrix sp.*	(Turroni*, et al.*, 2007)

Streptomyces spp. *Streptomyces sp.* *Variovorax paradoxus*	(Müller, 1950, Messini & Favilli, 1990, Sahin, *et al.*, 2008)
Achromobacter sp.? *Afipia* sp.? *Arthrobacter* sp.? *Ensifer adhaerens*? *Kribbela flavida*? *Lysobacter* sp.? *Polaromonas* sp.? *Rhodospirillum* sp? *Sinorhizobium* sp.? *Sphingomonas* sp.? *Stenotrophomonas* spp.? *Terrabacter* sp.? *Xanthomonas* sp.?	Bravo et al. 2013 to be submitted.

If we look at the scarce literature about the diversity of oxalotrophic bacteria in general (Table 1), we can observe that *Streptomyces* have been recognized as oxalotrophs since long (Müller, 1950; Messini et al. 1990; Sahin, 2008). The last part of the table shows the new oxalotrophic bacteria found in tropical forest (chapter 4), or characterized as active (chapter 3) in this study.

A new method was developed in this work to carry out studies on fungi-bacteria interactions implied in the OCP. The "inverted Petri dish method" was based on the fungal highway hypothesis, which explains the role of fungi, on the activity of oxalotrophic bacteria as a consequence of dispersal in unsaturated medium. The method has been confirmed to be suitable for the isolation of autochthonous couples fungi-bacteria related with the OCP. In the few section, it is presented a comparison performed bwteeen two oxalate-carbonate pathways observed at the tropical forest from India and Cameroon. This comparison include the analysis of abiotic and biotic factors needed to classify a dynamic OCP system.

A comparison between sites studied:

The oxalate-carbonate pathway and oxalotrophic bacteria: A comparison between tropical forest from India and Cameroon

Authors:

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Abstract

The formation of calcite in carbonate-free acidic soils through the biological degradation of oxalate can lead to a potential mechanism for terrestrial long-term carbon sequestration. This pathway, known as the oxalate-carbonate pathway (OCP), links the bacterial catabolism of oxalate (oxalotrophy) to the indirect production of carbonate by the alkalinization of the soil. In this study, the correlation between pedological variables and microbiological proxies in two different geographical areas was assessed. In Cameroon the study was carried out around the oxalogenic tree *Milicia excelsa*, which has been used in the past as a model to study the geological aspects of the OCP. In the case of India, the presence of oxalogenic trees was verified in the field. One individual of *Terminalia bellirica* was selected from the initial screening. In both cases soil samples were taken under the oxalogenic tree and in a control soil that was not influenced by it. Several biochemical and physicochemical parameters were studied in the field (pH and carbonate presence) and in the laboratory (soil pH, total organic carbon, and carbonate). Those were correlated with the abundance of oxalotrophic and total bacteria in soil measured by quantitative PCR (qPCR) using the frc and the 16S rRNA genes, respectively. The qPCR results from both sites indicated that oxalotrophic bacteria are ubiquitous, especially in the upper soil horizons. However, the presence of an oxalogenic tree influenced positively the abundance of oxalotrophic bacteria. Moreover, a correlation between frc qPCR and soil characteristics (particularly carbonate) was obtained for the soil influenced by the tree. These results demonstrate for the first time a correlation between the presence of carbonate and the abundance of oxalotrophic bacteria in situ. More broadly, they show the influence of vegetation on the abundance of specific bacterial guilds that can affect soil characteristics.

a. Introduction

The oxalate carbonate pathway (OCP) is a biogeochemical process of mineralization of calcium oxalate into secondary calcium carbonate due to the activity of soil oxalotrophic bacteria (Braissant, et al., 2004). Calcium carbonate precipitation cannot occur spontaneously at a pH below 8.4 at 25°C, and 1 atm. (Verrecchia, et al., 2006). However, bacterial oxalotrophic activity has been shown to lead to an increase of local soil pH in initially acidic soils, allowing carbonate precipitation (Braissant, et al., 2002, Martin, et al., 2012). The spontaneous oxidation of oxalate is impossible because of the high activation energy required, and so, any metal oxalate can be considered as a compound in a metastable equilibrium (Verrecchia, 1990). In previous studies we have demonstrated that active oxalotrophic bacteria in natural ecosystems (Bravo, et al., 2013) could be involved in the lack of geological records of oxalate in spite of its ubiquitous presence and important production by plants (Franceschi & Nakata, 2005) and fungi (Gentile, 1954, Dutton & Evans, 1996) in the whole geo-biosphere.

The activity of the OCP has important implications. The transformation of oxalate into carbonate causes a modification of the ion mobility (Graustein, et al., 1977), having a direct influence on soil fertility and on bioavailability of elements such as Ca, K, Na, Fe (Cole, et al., 1953) as well as, in detoxification of Al in soils (Klug & Horst, 2010). In addition, the existence of the OCP in a soil formed on calcium carbonate-free bedrock, leads to a net carbon sequestration (Cailleau, et al., 2004). In this case, atmospheric CO_2 fixed through the photosynthetic activity of plants forms the carbon skeleton of oxalate (Nuss & Loewus, 1978). At a first stage of the pathway, the oxalate produced plays a role in calcium regulation, specialized defense mechanisms and heavy metal detoxification (Prior J & D, 1992, Franceschi & Nakata, 2005). Oxalate can also be produced or released by the degradation of vegetal tissues due to the activity of saprophytic fungi (Cromack, 1972; Guggiari, et al., 2011). This has been remarked comparing continental weathering and mineral neogenesis (Verrecchia, et al., 1993, Burford, et al., 2003). The bacterial respiration of oxalate recycles the carbon atoms from oxalate leading to the formation of carbonate and hydrogen-carbonate ions in the soil solution. When calcium is available, calcium carbonate precipitates forming a geologically stable carbon sink from a biological reservoir of organic matter (Verrecchia, et al., 2006).

Moreover, an increasing number of studies document that oxalotrophic bacteria can use calcium oxalate as sole carbon and energy source (Tamer & Aragno, 1980, Aragno & Schlegel, 1991, Sahin, 2003, Bravo, et al., 2011). To study this functional group of bacteria, several methods have been proposed. Culturing methods using selective medium have shown to be useful to collect oxalotrophic bacteria (Tamer & Aragno, 1980, Braissant, et al., 2002). Nevertheless the limitation of culturing methods to assess the biodiversity and abundance of oxalotrophs *in situ* are well known. Therefore, the use of genes coding for enzymes involved in the catabolism of oxalate as molecular markers is a better-suited strategy. Primers designed to amplify the gene *frc* (codifying for the formyl-CoA transferase) have already been used to assess diversity and abundance of oxalotrophic bacteria in situ (Khammar, et al., 2009) and recently, we have used the *frc* gene to identify active oxalotrophic bacteria *in situ* (Bravo, et al., 2013). Nonetheless, the molecular evaluation of oxalotrophs is only one component of the pathway.

Although the biotransformation of oxalate into carbonate and its consequences on the environment have been studied in temperate forest (Cromack, et al., 1977), Mediterranean to semi-arid calcrete environments (Verrecchia, 1990, Verrecchia & Dumont, 1996), and tropical soils (Braissant, et al., 2004, Cailleau, et al., 2004, Bravo, et al., 2013), a comparison of biotic (oxalotrophic activity) and abiotic (physicochemical soil features) components, that contribute to stimulate the pathway occurring in tropical biomes has not been done in the past. Those biomes could represent the major reservoir of natural OCP systems contributing to mitigate elevated concentrations of atmospheric CO_2.

In consequence, the aim of the present study was on the one hand to establish the occurrence of the OCP in India, and on the second hand to highlight the relationship between oxalotrophic and total bacterial abundance, with soil pH_{H2O}, carbonate content, and total organic carbon in soil, by comparing a well-established OCP system (*Milicia excelsa*) occurring in Cameroon, with a novel one (*Terminalia bellirica*) found in a forest from India. The questions leading this study were: i) Are oxalogenic trees and oxalotrophic bacteria present in India? ii) Is there a higher proportion of oxalotrophic bacteria under an oxalogenic tree than under soil not influenced by the tree? and iii) is the abundance of the oxalotrophic bacteria correlated with environmental parameters (i.e. soil pH, carbonate)? To answer these questions, samples from two selected trees were collected and assessed for the activity of the OCP. Soil pH was measured on the site to confirm alkalinization due to the activity of oxalotrophic bacteria. The oxalogenic tree *Terminalia bellirica* was selected in the tropical forest from India. In Cameroon an individual of the known biomineralizing tree

Milicia excelsa was selected. Soil samples were taken under the tree canopy (soil influenced by the tree) and in a control soil (not influenced). DNA was extracted from those samples quantifying total and oxalotrophic bacteria by quantitative PCR (qPCR). A statistical approach was carried out to compare both biotic and abiotic compounds at each OCP system. The importance of oxalotrophic bacteria and determinant soil physicochemical factors, as well as the efficiency of an OCP in tropical soils are discussed.

b. Materials and Methods

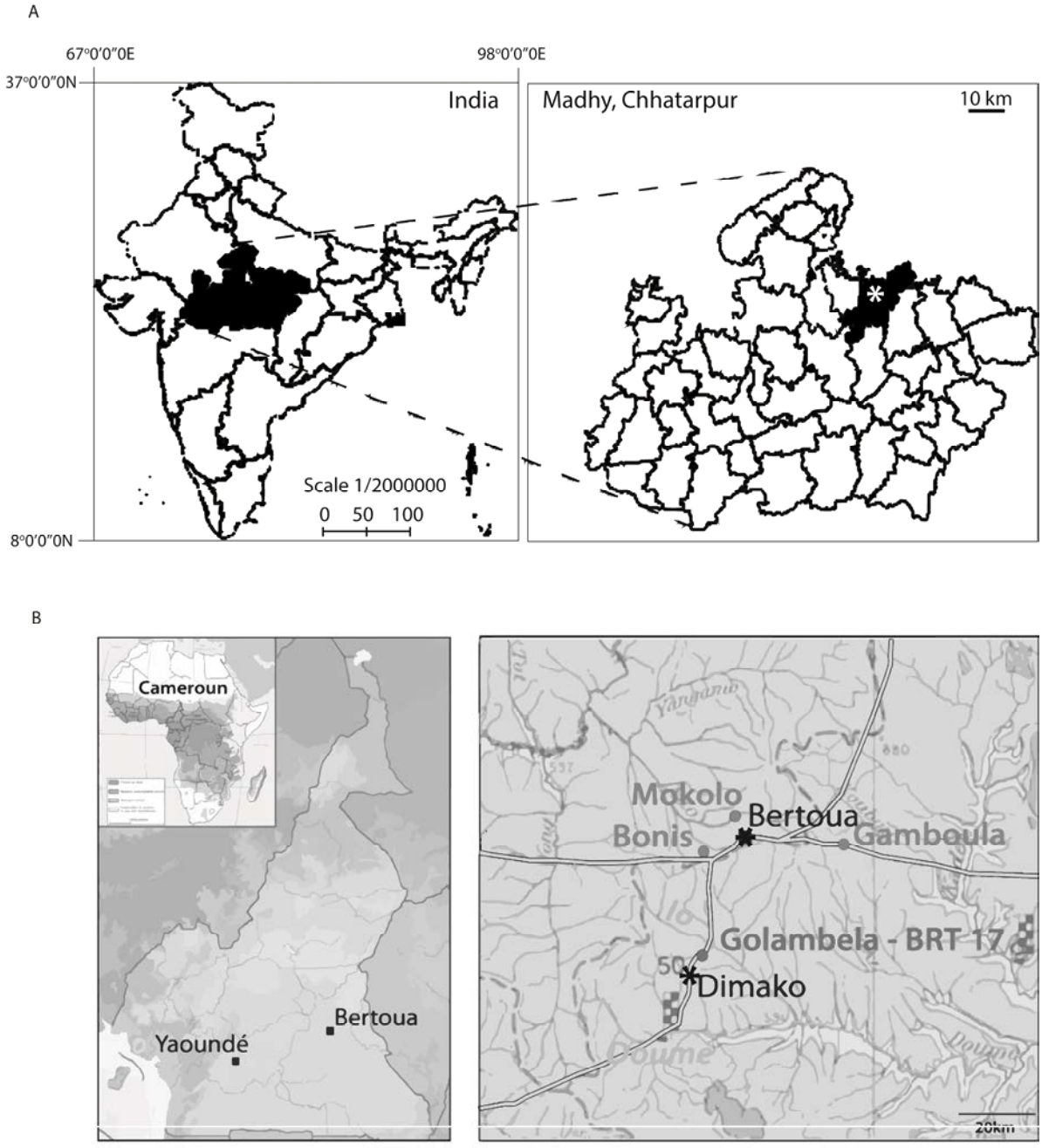
i. Sampling sites

The sampling sites were tropical soils from India and Cameroon. In order to find calcium carbonate accumulations in Indian soils, the prospection of the sampling site was made based on the following three criteria: i) existence of oxalate producing vegetation, ii) presence of oxalotrophic bacteria, and iii) climatic conditions including a dry season allowing the accumulation of calcite (Braissant, et al., 2004, Cailleau, et al., 2004). A flat zone was favored since this decreases carbonate leaching in the soil.

Indian forest was located 25 km south from the city of Khajuraho (Madhya Pradesh State), in the North Panna Tiger Reserve forest (24°38'18"N / 79° 51'9"E). This region belongs to the Indo-gangetic area (See supplementary Fig 1A.), with a soil type corresponding to a Luvic Cambisol formed over granite bedrock (Peel, et al., 2007). The climate was defined as a tropical monsoon with three seasons (summer, winter and monsoon) (IUSS, 2006), with a total annual rainfall higher than 1000 mm and a mean annual temperature of around 23°C. An exploratory phase was carried out to find potentially interesting tree specimens for the detailed analyses. During the exploratory phase two tests were applied for the samples in situ: i) the presence of carbonates on tree tissues and soil was revealed by effervescence after the addition of 10% HCl; and ii) soil pH near the tree trunk and far from it was measured using the colorimetric method of the HELLIGE® Soil Reaction pH Tester (Ben Meadows, USA). Two pedological profiles were prepared, according to previous studies (Cailleau, et al., 2004, Gobat, et al., 2004). The first one (Profile A) was located under the oxalogenic tree *Terminalia bellirica* (Bahera) up to 100 cm depth. The second one (control soil, Profile C) was obtained 14 meters away from the tree up to 100 cm depth as well. The control profile corresponded to soil without the influence of nearby trees.

Tree *Millicia excelsa* (Iroko) in Cameroon was located in an ancient syntectonic granite area at Golambela (4°25' N, 13°36' E; see supplementary Fig 1B). The climate was defined as a tropical monsoon as well as in India (IUSS, 2006), with a mean annual temperature around 24°C. The soil was classified as Ferralsol with secondary carbonates (Peel, et al., 2007). The diameter of the tree was 2.5±03 m and the age was estimated between 200 and 250 years. The soil below the hollow trunk contained considerable amounts of carbonate as it has been observed in the same plant species in Ivory Coast (i.e. c. 500 kg inside the tree and c. 1000 kg in surrounding soils(Braissant, et al., 2004), suggesting that an active OCP could be occurring. In this area, soil profiles A and C

were separated by 10 meters. Profile A was prepared up to 56 cm depth and Profile C up to 133 cm depth.



Supplementary Fig 1. Map of sampling sites in India (A), and Cameroon (B), indicated with an asterisk, where the OCP systems were assessed. The locations were classified as tropical forest with monsoon climate. Natural environment and flat topography were some of the most important criteria to assess the presence of the oxalate-carbonate pathway in those ecosystems.

ii. Soil samples

For molecular analysis, soil samples were taken in each profile (A and C, independently), at each sampled site. In India, 14 soil samples were collected from profile A and 13 samples from profile C. In the case of Cameroon, 11 soil samples were used from profile A and the same number from profile C. The samples (around 5 g) were immersed in sterile Falcon tubes (VWR, France), containing 5 mL of RNA Later solution (Qiagen), and stored at 4°C until processing. For pedological analysis, soil samples were taken every five centimeters up to one-meter depth. Different samples from tree tissues, as well as litter on both profiles were also collected.

iii. Pedological analyses

In order to demonstrate the influences of the abiotic factors from soil on the OCP, all samples of both profiles where characterized for the soil variables: soil pHH2O, total organic carbon, and carbonate. To measure soil pH_{H2O}, ten grams of 2 mm sieved soil was left in 25 ml of de-ionized water for 1 h and measured with an electrode (Metrohm, Switzerland). Before measuring pH soil particle sizes were defined by liquid laser granulometry (Mastersizer2000 with Hydro2000S, Malvern Instrument, UK). For that, organic matter from the samples was entirely oxidized by treatment with warmed H_2O_2 (up to 30%). To avoid flocculation of particles, sodium hexametaphosphate (40 g L^{-1}) was added. For each sample, three measurements were made. To measure calcium carbonate content in soil samples, a titration using sulfuric acid (0.5 N) and sodium hydroxide (0.5 N) on 1 g of crushed bulk sample was performed. Total organic carbon (TOC) was determined by pyrolysis in a Rock-Eval 6TM analyzer (Vinci technologies, France). For this 0.3 g of crushed soil were placed into an oven that increased temperature progressively to initially 300°C and then 550°C in a N_2 atmosphere. Carbon compounds were measured with both FID (H/C gas) and an on-line infrared cell (CO and CO_2).

iv. DNA extraction

DNA extractions were performed with a Fast DNA Spin Kit for Soil (Bio 101 Sytems, Q-Biogene, Switzerland) following the manufacture's guidelines. Total DNA of soil was extracted from 0.1 g of soil samples. Then, 450 μL of DNA lysate was purified using 450 μL of binding matrix. DNA was separated in a final volume of 80 μL in a DES solution. No specific humic acids extraction method was performed, nonetheless, after measuring the final DNA concentration by spectrophotometry (Nanodrope ND-1000, Thermo Scientific, USA), dilutions

on MiliQ sterile water were made in order to minimize contamination disturbing the amplification (final DNA concentration of 3±1 ng μL^{-1}).

v. Quantitative PCR (qPCR)

In order to confirm the presence and estimate the abundance of oxalotrophic bacteria, quantitative PCR (qPCR) on the *frc* gene (Khammar, et al., 2009) and on the V3 region of the 16S rRNA gene (Muyzer, et al., 1993) were performed in a Rotor-Gene 3000-A (Corbett Life Science, Australia). The qPCRs were performed by triplicate. For the frc gene amplification (in a total volume of 10 µL), two µL of the diluted DNA were mixed with 0.25 µL of the primers frc171-F (5'-CTSTAYTTCACSATGCTSAAC-3') and frc306-R (5'-GDSAAGCCCATVCGRTC-3') (Final concentration of 1.25µM). In addition, 5 µL of the SYBR Green PCR kit (Quantitect, Qiagen, Germany) was added. The final volume of the mix of 10 µL was completed adding RNAase free water. The frc-qPCR program consisted in enzyme activation at 95°C during 15 min followed by 40 cycles of denaturation at 95°C during 10 sec, annealing at 56°C for 60 sec, and extension at 72°C during 20 sec. A standard curve and the Ct value were established with a serial dilution of known copy numbers of the *frc* gene from DNA isolated of *Oxalicibacterium flavum* strain NEU98 (DSM 15506) cloned into a pGem T Vector.

To amplify the V3 region of the 16S rRNA gene the qPCR mix contained 2 µL of diluted DNA samples, 0.3 µL of each of the primers 338-F (5'-ACTCCTACGGGAGGCAGCAG-3'), 520-R (5'-ATTACCGCGGCTGCTGG-3') in a final concentration of 0.3 µM, and 5 µL of SYBR Green PCR kit. The final mix volume of 10 µL was completed with RNAase free water. The qPCR program for the V3 region consisted in the same protocol used by the *frc* gene, except that the annealing step was modified at 55°C during 15 sec. In this case, the standard curve and Ct value were generated with a serial dilution of known copy numbers of the 16S rRNA gene from DNA isolated of *Bacillus subtilis* strain 168. In order to validate the purity of the amplified products in both cases, a melting analysis was run from 72°C to 95°C to check the fluorescence fall at the fusion temperature.

vi. Data analysis

In order to bring out the occurrence of the OCP in India and to compare it with the OCP in Cameroon, correlation coefficients were calculated between microbial and soil physical variables occurring at soil samples recovered from *T. bellirica* and *M. excelsa* at profile A. In addition, a principal components analysis (PCA) was performed to understand the critical variable factors affecting the OCP at each sampled site in profile A. The qPCRs of *frc* and 16S rRNA genes, as well as, soil pH, carbonate, TOC, and depth were taken into account as variables for the statistical analyisis. The correlations were calculated using SigmaPlot (version 11.0) and the PCA analysis was made using the freeware R statistics.

c. Results

i. Oxalogenic trees and the OCP system in India

Thirty-five out of the 65 tree species listed by the Forest service from the state of Mhadya Pradesh could be found in the field and assessed for a potentially active OCP (Table 1). Four out 65 species showed the presence of carbonates in the tree tissues or soil nearby and an alkalinization of the soil near the tree trunk. The trees belonged to different botanical families (i.e. *Terminalia bellirica* belonged to the family *Combretaceae*). Between those species, *T. bellirica* present an economical and cultural value, used to produce therapeutic brewages as an alternative treatment against coronary disorders (Phulwaria, et al., 2012).

Table 1. List of tree species showing the presence of carbonates and an alkaline soil pH near the trunk found during the exploratory phase in India. Species showing an increase of pH in the surrounding soil and a positive reaction to the HCl test are indicated as (+)

Family	Genus specie	Vernacular name	Results HCl and pH test
Fabaceae	*Acacia leucophloea*	Raijunja	-
Rubiaceae	*Adina cordifolia*	Haldu	-
Combretaceae	*Anogeissus acuminata*	Kardhaie	-
Rutaceae	*Aegle marmelos*	Bel	+
Euphorbiaceae	*Bridelia retusa*	Kasai	-
Burseraceae	*Boswellia serrata*	Salei	-
Anacardiaceae	*Buchanania lanzan*	Acharwa	+
Fabaceae	*Butea monosperma*	Palas	-
Ebenaceae	*Diospyros spp*	Tendu	+
Rutaceae	*Feronia limonia*	Kainthi	-
Moraceae	*Ficus religiosa*	Peepal	-
Ulmaceae	*Holoptelea integrifolia*	Chilla	-
Lythraceae	*Lagerstroemia parviflora*	Seja	-
Sapotaceae	*Madhuca indica*	Mahua	-
Annonaceae	*Milusa tomentosa*	Kari	-

	Mitragyna parvifolia		-
Rubiaceae	Var. *M. macrophylla*	Kam	
Fabaceae	*Pterocarpus marsupium*	Visa	-
	Salmalia malabarica		-
Bombacaceae	Syn. *Bombax ceiba*	Semar	
Fabaceae	*Tamarindus indica*	Emli	-
Lamiaceae	*Tectona grandis*	Sagoon	-
Combretaceae	*Terminalia bellirica*	Saja; Bahera; Koha	+

Due to the presence of carbonate after HCl test, the specimen of that species was selected for detailed soil and microbiological analyses. *T. bellirica* was the largest specimen found in the area, which was subjected to lodging and have accomplish the criteria described in the methods. The tree studied was approximately 20 m high with a diameter of 79 cm. Its age was estimated on around 50 years old (this tree species is reported to live up to 100 years). The canopy covered an area of 13 m around the trunk. Soil pH near the tree trunk was 8, while pH from the soil at a distance of 10 m from the trunk was 4.5. A strong HCl reaction was observed for the tree bark and the superficial soil near the trunk.

ii. Influence of T. bellirica (India) on soil microbial communities

The figure 1A shows the results of the soil composition at profile A that was prepared under the influence of the oxalate-producing tree *T. bellirica*. At that profile, three horizons were identified. The upper one (horizon A) was 5 cm wide, biomacro-structurated and more rich in organic matter than the other two. The second horizon (E) was characterized as an elluvic horizon, with visible oxide-reduction spots and lower clay content. The limit between this horizon and the next one (horizon B), was diffused and corresponded to an augmentation of the clay content. The third horizon, (B), was more compact and richer in clay and was therefore qualified as an accumulation horizon. Roots were observed at four different levels: thin and medium roots (around 0.2-1 cm width) in the first horizon; big roots (around 4 cm width) between 7 to 15 cm depth; thin hairy roots (around 0.2-0.8 cm width) between 20 and 30 cm depth; and, medium and big roots (around 2-6 cm width) in the limit of the two deepest horizons (from 40 to 50 cm depth). The control soil (Profile C) was located 14 m away from the studied oxalate-producing tree and it was also defined as a Luvic Cambisol (Fig. 1A). In this control profile, three horizons were also defined with a limit at 10 cm depth and a transition limit around 40 cm depth. On this profile, there were no specific areas with roots. Nonetheless, thin hairy roots were observed all along the profile.

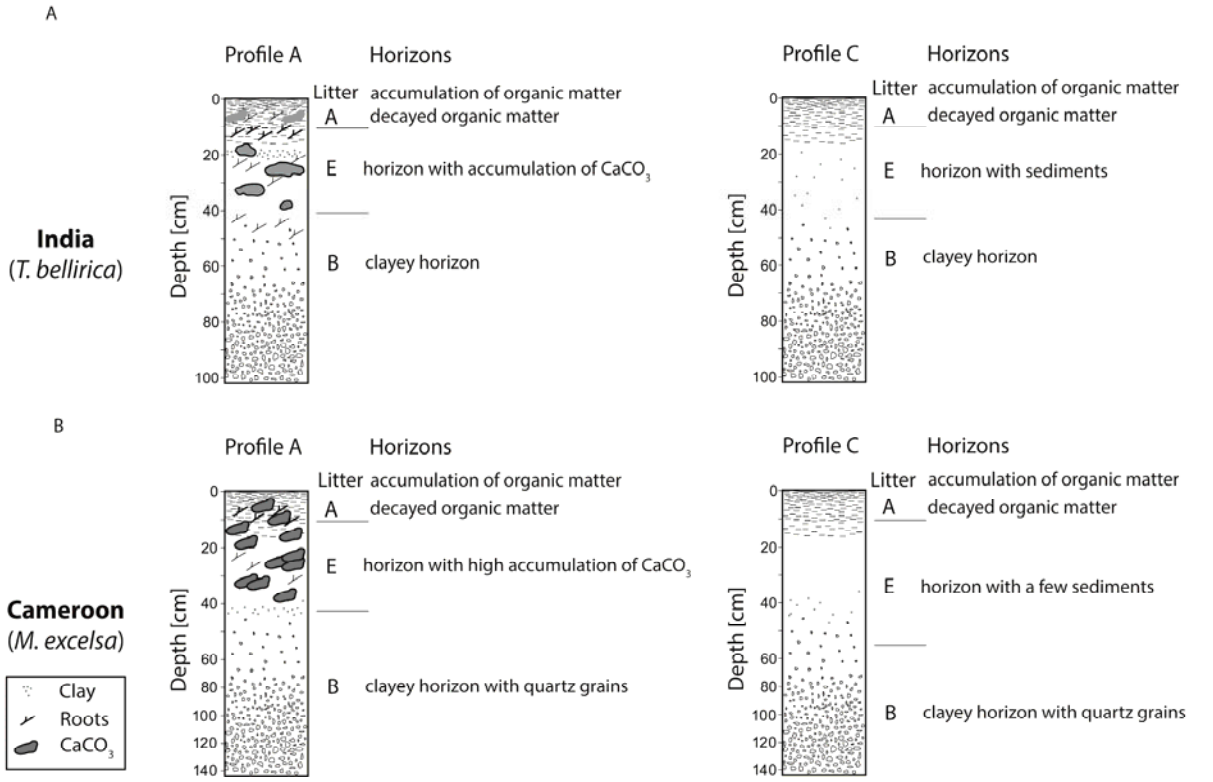


Fig 1. Description of soil profiles in (**A**) India and (**B**) Cameroon. Profile A prepared at one side of the tree (*T. bellirica* or *M. excelsa*). Profile C was the control soil without any influence of surrounding trees.

Real-time amplification (qPCR) for the *frc* gene and the V3 region of the 16S rRNA gene was successful for all the soil samples at profile A (Fig. 2A & B). At that profile, the tendency observed in both oxalotrophic and total communities by the gen markers used was very similar, with a high number of copies at the first layer in horizon A, decreasing considerably in depth. However, the number of copies obtained for total bacterial community was ten times higher than the number of copies found for oxalotrophic bacterial community. Interestingly, the abundance of oxalotrophic and total bacteria was very high on the upper part of the profile A (3.1E+08 and 1.7E+10 copies per g of soil, respectively), and then decreased considerably with depth. Moreover, high abundances were also observed in three other horizon sections of the profile, at 10-15cm, 20-25cm and 50-55cm depth, respectively. In profile C qPCR results for both *frc* and 16S rRNA genes were also well correlated. The sample from the very top of the profile C (litter) has not representative communities, except by the oxalotrophic communtie at soil samples of 17 cm which then decreased dramatically for the rest of the profile, being close to the detection limit for all but the sample at 65 to 70 cm depth.

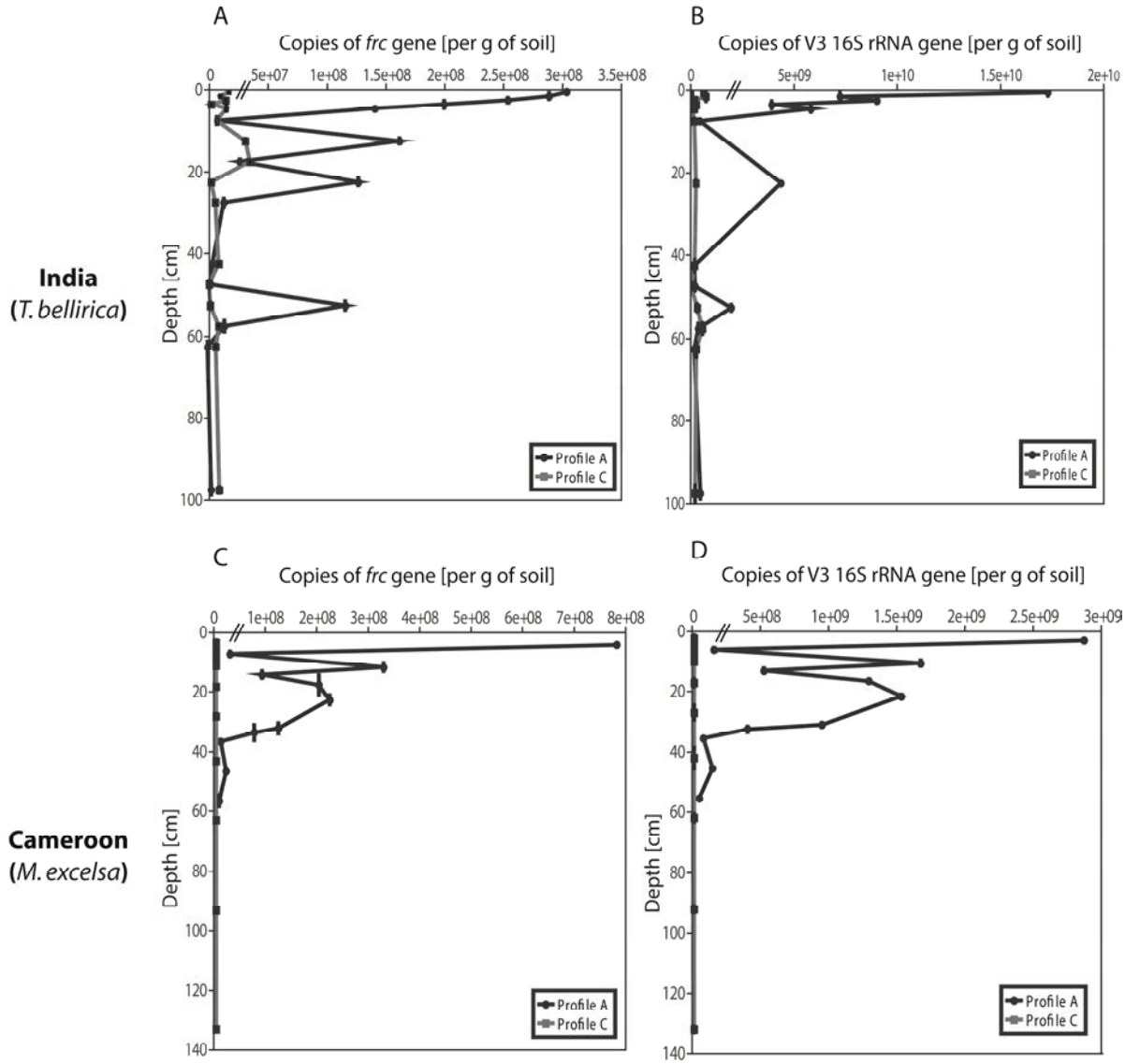


Fig 2. qPCR data of *frc* and V3 - 16S rRNA genes from India and Cameroon. The vertical lanes correspond to the standard deviation of the replicates. (**A**) qPCR of *frc* gene performed with soil samples from India. (**B**) qPCR of region V3 16S rRNA gene of the soil samples collected in India. (**C**) qPCR of *frc* gene in profiles A and C from Cameroon, and (**D**) qPCR of the region V3 of the 16S rRNA gene performed in soil samples from Cameroon. In all the cases both profiles A (in black) and C (in gray) are shown. ("//"= Scale of gene copies adjusted to the graphic).

The physical soil parameters are shown alongside qPCR results (Table 2). Soil pH_{H2O} for profile A was alkaline near the surface (pH max of 8.4 at 0 and 5 cm depth), but near to neutrality with depth. An alkalinization was noticeable also between 20 to 25 cm and 30 to 35 cm depth. Soil pH_{H2O} for profile C was acidic in the upper part (pH closer to 6 after the first 5 cm) increasing relatively in a constant rate within the depth to reach an alkaline level of 8.6 at 100 cm depth. Furthermore, calcium carbonate content was 1.5 % on the upper part of the profile A, but then it has decreased rapidly to 0.1 % at 75 cm depth. A small augmentation of carbonate is noticeable at

95 - 100 cm depth with 0.6 % of carbonate content. In addition, carbonate content along the profile C was very low (less than 1%, data not shown), even if it seemed to increase smoothly with the depth.

Table 2. Microbiological and abiotic variables compared between profile A from India and Cameroon. (*frc*) *frc* gene quantification; (V3) V3 region of the 16S mRNA gene; (pH_{H2O}) Soil pH value; (TOC%) total organic carbon; (Depth) Depth according to sampling protocol.

Origin	Sample	qPCR *frc*	qPCR V3	pH_{H2O}	$CaCO_3$ (% d.w.)	TOC%	Depth (cm)
India	A1	3.07E+08	1.73E+10	8.37	1.5	1.77	0 - 1
	A2	2.92E+08	7.23E+09	8.37	1.5	1.77	1 - 2
	A3	2.57E+08	9.02E+09	8.37	1.5	1.77	2 - 3
	A4	2.03E+08	3.92E+09	8.37	1.5	1.77	3 - 4
	A5	1.44E+08	5.82E+09	8.37	1.5	1.77	4 - 5
	A9	1.65E+08	4.38E+09	7.87	0.8	0.73	20 - 25
	A13	2.92E+07	1.84E+08	7.64	0.7	0.58	40 - 45
	A15	1.55E+07	1.97E+09	7.23	0.2	0.43	50 - 55
	A17	4.05E+06	1.74E+08	7.04	0.1	0.48	60 - 65
	A19	0.00E+00	0.00E+00	6.94	0.1	0.52	70-75
	A24	1.19E+08	4.73E+08	7.14	0.6	0.31	95 - 100
Cameroon	A1	7.78E+08	2.86E+09	7.75	4.3	6.68	1-5
	A2	2.68E+07	1.44E+08	7.8	4.0	5.78	6-8
	A3	3.25E+08	1.66E+09	*7.82*	*2.0*	3.23	10-11
	A4	8.92E+07	5.11E+08	*7.84*	*0.5*	2.19	12-14
	A5	1.99E+08	1.28E+09	*7.82*	*1.3*	*1.54*	16-17
	A6	2.20E+08	1.52E+09	7.74	1.3	0.8	20-23
	A7	1.21E+08	9.38E+08	7.5	0.2	0.75	30-32
	A8	7.41E+07	3.90E+08	*7.44*	*0.5*	*0.74*	32-33
	A9	8.89E+06	6.79E+07	7.37	0.8	0.72	35-36
	A10	1.92E+07	1.36E+08	7.52	0.0	0.68	45-46
	A11	5.59E+06	3.58E+07	7.62	0.7	0.73	55-56

iii. Influence of M. excelsa (Cameroon) on soil microbial communities

The figure 1B shows the results of the soil composition at profile A, that was prepared under the influence of the oxalate-producing tree *M. excelsa*, in Cameroon. At simple field observations it was possible to confirm the presence of secondary carbonate. The horizons were composed by considerable amount of calcium carbonate (4.3% dry weight) at the upper part of the profile A (horizon A). Roots have been observed at the first 14 cm depth and thin roots upt to 33 cm depth (horizon E). The horizon B was characterized by clayed with quartz grains. The control soil (Profile C) was located at 10 m away of the oxalogenic tree. The horizons were composed by a first layer of organic matter (horizon A), followed by an intermediate zone of few carbonate depositions (horizon E) and progressive deposition of clay with quartz grains (Fig. 1B).

At microbiological level, the qPCR amplicons for both *frc* and V3 markers have shown a similar tendency (Fig. 2C & D) observed in the composition of bacterial communities at profile A and C in India. In profile C was not detected oxalotrophic nor total communities.

The physical soil parameters for profile A Cameroon (Table 2), shown in profile A a soil pH parameter with a weak tendency through alkalinity near to the surface (pH max of 7.75 at 1 and 5cm depth, respectively), but with a few tendency to increase alkalinity with depth (pH 7.84 between 12 and 14 cm). An alkalinization was noticeable also between 16 to 17 cm and 20 to 23 cm depth (pH 7.82 and 7.74, respectively). Soil pH in profile C was acidic at the upper part (pH closer to 5 after the first 10 cm) and it maintained relatively constant with depth (data not shown). Interestingly, calcium carbonate content in profile A decrease with a similar distribution as has been obtained for qPCR data from oxalotrophic and total bacteria, showing peaks of activity at three range of distances (6 to 8, then 16 to 17, and 35 to 36 cm depth, respectively), with a high abundance of oxalotrophic bacteria on the upper part of the profile where 4.3 % of calcium carbonate was found.

iv. Correlation of microbiological and soil analyses

Correlation coefficients between the data are shown in Figure 3. For profile A of the OCP system assessed in India (Fig 3A), microbiological variables were very well correlated between with soil traits such as pH, carbonate and TOC. For this analysis depth was also considered as a variable, which showed a correlation with carbonate content. Even though the microbiological variables were well correlated together, they were not correlated with the soil traits within the control soil.

The correlation coefficients obtained for Cameroon soil samples from profile A (Fig 3B) shows that carbonate precipitation at the first centimeters reduce the regression value. Therefore the correlation between biotic and abiotic is less significant.

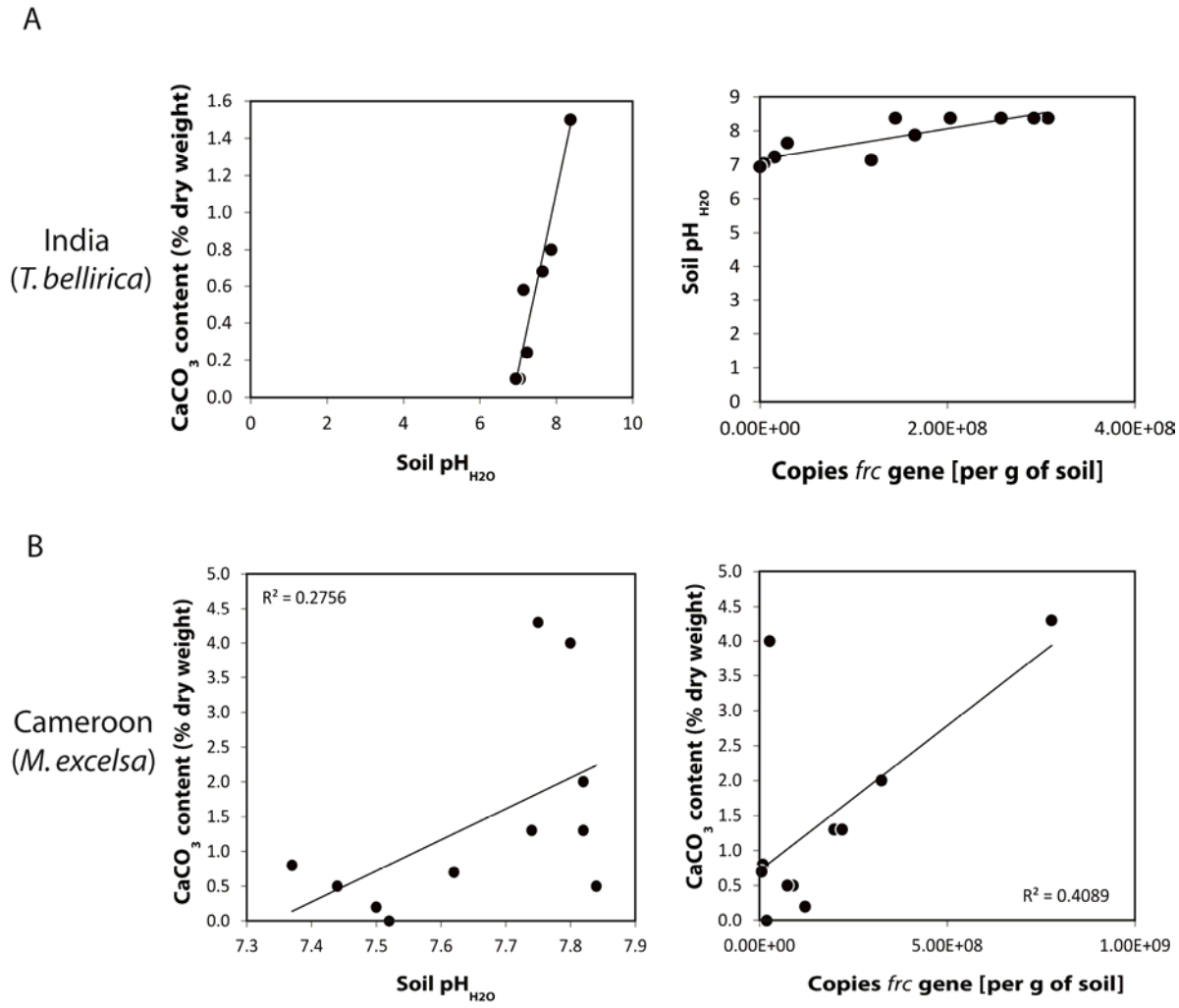


Fig 3. Scatterplots of carbonate content in % of dry weight vs. pH_{H2O} and an abiotic factor Soil pH or $CaCO_3$ content vs. qPCR data of oxalotrophic communities measured in both OCP systems (*T. bellirica* and *M. excelsa* from India and Cameroon, respectively) at profile A. In India (**A**), the regression line calculated using these data is characterized by a high r2 = 0.9566. Therefore, the regression line shows that there is a relationship between soil pH_{H2O} and carbonate content, as well as, between soil pH and frc gene copies (oxalotrophic community in profile A). In Cameroon (**B**), the regression line calculated shows a low r^2 = 0.2756. Thus, there is a weak correlation between carbonate content and soil pH, as well as, between carbonate and *frc* gene copies.

v. Variables factor analysis

Figure 4 shows the statistical analysis of a set of 5 observations or correlated variables. For instance in India (Fig 4A), the abiotic factors are well correlated in comparison with the biotic factors (qPCRs of *frc* and V3-16S rRNA genes). In addition, in the case of Cameroon (Fig 4B), the correlation is better within the intraspecific treatments (in this case abiotic or biotic), than the interspecific correlation between those factors. Interestingly, both OCP systems seem to have

more or less the same correlation when is observed the patron of decreasing quantities of each factor within depth in profile A.

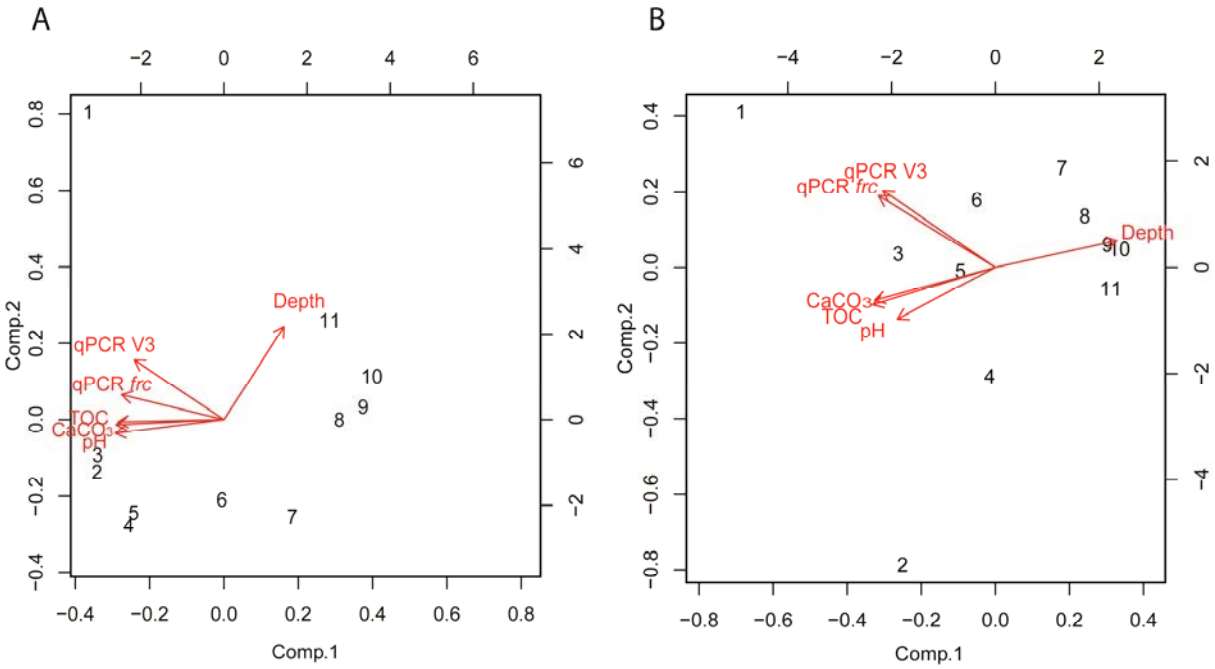


Fig 4. Variables factor map or principal component analysis (PCA) performed with soil samples from profile A in Indian (**A**) and Cameroon (**B**) OCP systems (T. bellirica, and M. excelsa, respectively). The map shows the correlation between soil pHH2O, CaCO3 content and total organic carbon (TOC), with the microbiological factors (qPCRs of frc and V3-16S rRNA genes). The analysis in component 2 explains in 94.60% and 92.85% the correlation between these factors.

d. Discussion

We highlight the occurrence of the OCP in oxalogenic trees of *Terminalia bellirica* found at tropical forest in India by the accomplishments of the requirements cited at the introduction for an OCP system. Regarding the importance of the tree selected in the use that the local communities give to it, we can point out some reasons that make attractive the further use of this tree. For instance, it has been reported that its leaves were used as animal forage (Phulwaria, et al., 2012). Furthermore, their fruits have been used in traditional medicine and the seeds have been shown to produce up to 40% of oil, which can meet the standards for the production of biofuels (Reddy, et al., 2011). In addition to that, our investigation shows that besides the cited properties, this particular tree species induces a biomineralizing reaction that should be taken into account within the climate change and atmospheric CO_2 issues. For example, the specimen could be used for further forestation programs around trees with elevate agronomic importance. In that way *T.*

bellirica will improve not only on soil quality due the activation of OCP, but also by being a potential source of additional income from derived products.

Moreover, this study has shown by the analysis of the qPCRs performed using soil samples from India and Cameroon that oxalotrophic bacteria are ubiquitous along the OCP systems assessed. However, it has been confirmed that oxalotrophic bacterial communities are clearly stimulated by the presence of an oxalogenic tree (Fig 2). In consequence, around the oxalogenic tree, oxalotrophic bacteria will favor the precipitation of calcium carbonate. This can be explained given a positive correlation calculated between qPCR of the gene *frc* and abiotic soil traits, especially looking the carbonate content in soil. These correlations prove that the OCP may occur actively in the studied systems. Both *frc* and V3 region of the 16S mRNA gen amplifications and quantification have shown similar distribution in depth, and were well correlated in *T. bellirica* (coefficient correlation: 0.921) and in *M. excelsa* (coefficient correlation: 0.898) systems. The fact that higher concentrations of carbonate were found in those systems at the first 20 cm is correlated with the presence of oxalotrophic bacteria, which in both cases could represent a bigger part of the total community, especially in the strategic parts of the soil where organic matter is massively released. Real time qPCRs of the frc gene in profile A from India and Cameroon (Fig 2A & C) have shown that may occurs certain hotpots of oxalate releasing through the root system disposed mainly in horizons A and E. The fact that a copious number of roots were found in the soil profile is an indication that microniches of oxalate could be increasing the oxalotrophic communities in the surrounding environment.

Almost each picks of presence of bacteria (for both *frc* gene and V3 region of 16S mRNA) can be related to specific area on soil description. Furthermore, the ration *frc*/V3 is higher for each of this peaks indicating that they might be directly related to the oxalate producing plant or the release of this compound in soil. If that is the case, in terms of oxalate sources, we could describe two main source of oxalate in a vertical soil profile: (i) the litter, in which saprophytic activity of fungi and bacteria will generate a gradient of oxalate upper horizon of soil, and (ii) the net of root systems distributed above the oxalogenic tree. In terms of oxalotrophic bacteria distribution, the profile under the oxalogenic tree (profile A) could be divided into three parts that will be discussed: (i) the upper part of the profiles, from 0 to 10 cm depth where litter organic matter integration is occurring, (ii) the high density roots area, among the first 60cm of the profile, where rhizospheric activity and roots decay are taking place, and (iii) the lowest part where no increase of bacteria is observed.

i. The upper part of the soil profile

The litter is a high biological activity zone (Wanner, 1970, Aerts, 1997). The maximal potential of soil at this horizon to sequester carbon is determined by intrinsic abiotic soil factors, such as topography, mineralogy and texture (Jastrow, et al., 2007), thus, soil carbon dynamics at this level is driven by oxalotrophs and their many interactions with their surrounding environment. In tropical soil, termites (and their associated organisms) are known to be one of the major player in organic matter fragmentation and integration in soil (Bachelier, 1978). Despite the high aerial vegetal production in this climate, the termites activity added to a high microbial activity leads to a rapid turn-over of the organic matter in soil (Cailleau, et al., 2011). Therefore, we cannot sub estimate the role of the ecosystem engineers residing the horizon A that affect carbon sequestration through carbon consumption modifying soil physical structure. Thus, the low rate of organic matter measured in this studied soils is not so unexpected, since earthworms, ants, and termites promotes as well carbon sequestration (De Deyn, et al., 2008) allowing such a shift.

The oxalotrophic activity close to surface highlights the litter degrading fungi and bacteria, that are in continuous contact through the fungal highways net (Bravo, et al., 2013), increasing oxalotrophic activity at that horizon. The high density of oxalotrophic and total bacteria in the top of the profile and decreasing rapidly within ten first centimeters (Fig 2A & B) is typical of an integration process of organic matter from the tree litter in tropical soils (Wanner, 1970, Six, et al., 2000). The profile A is at 50 cm from the center of the tree and therefore has a high rate of litter coming from the upper part of the studied tree *Terminalia bellirica*, proved to be oxalogenic. The same has been obersved in *M. excelsa*, in Cameroon. Litter fall enriched with calcium oxalate crystals is degraded by soil fauna, microbiota releasing considerable amounts of oxalate (as has been quantified at soil samples from *M. excels*a, data not shown). We should keep in mind that saprophytic fungi can also mobilize as well as produce oxalate, stabling several ecological relationships with oxalotrophic bacteria (Bravo et al., 2013). The good correlation coefficient of *frc* gene concentration compare to organic matter quantification (0.888) and to carbonate concentration (0.887) seems to match with the high rate of oxalotrophic bacteria among the total community in the horizon A. The control profile (profile C in Fig 2A & B) is 14m away from the studied tree. At that profile, the first horizon shows by qPCR data, that no litter integration process occurs since very few oxalotrophic bacteria were detected. The unexpected high rate of *frc* community at 15 cm depth in India and total bacteria at 5 cm depth of the profile C can be explained by lateral transport of organic matter enriched in oxalate due to stochastic processes

occurring nearby (i.e. wind transportation of litter containing calcium oxalate within the control profile).

ii. High density roots area

The organic matter integration in soil also occurs at a strong level in rhizospheric area where exchanges between roots, soils particles and soil microbiota are very intense. Roots released large amount of organic molecules, including protons and organic acids, modifying the very close roots environment and allowing a high heterotrophic microbial activity in its neighborhood (Gobat, et al., 2004). Organic acids efflux from the cytoplasm of roots cells to the soil solution can be done through two pathways: a slow passive diffusion throw the membrane or a plasma membrane channel protein. The direction of transport of organic acid is controlled by the electrochemical potential gradient across the membrane (mainly controlled by a proton ATP-ase) (Jones, 1998). The faith of plant roots exudation (including oxalate and other organic acids) can be divided in tree categories: uptakes by the rhisospheric microbial biomass (bacteria and fungi) implied in the pathway, sorption and fixation on soil particles (Fe/Al oxide-hydroxides and clays, since oxalotrophic bacteria is implicate in soil Fe/Al detoxification) and finaly, leaching in the direction of the lowest point.

The presence of oxalotrophic bacteria in depth is most likely related to rhizosphere and mycorhizosphere microzones. The point with the highest *frc* gene concentration at 20 – 40 cm profile A carried out in India and Cameroon (Fig. 2A & B) is related to a depth where small hairy roots are more concentrated (profiles A Fig 1A & B). It is well known that the hairy zones in roots are the most privileged exchange places within roots dynamic (Six, et al., 2000).

iii. The lowest part of the profile

The lowest part of profile A is characterized by the presence of multiple sources of protons and anions in soils thorugh lixiviation of ionic species (i.e. deprotonation of CO_2, organics acids, mineral weathering, and acid atmospheric deposition; van Breemen & Mulder, 1986). In addition, the assimilation of cations and anions in the sites studied could be explained by roots interchange, maintaining the electrical neutrality across the rhizoplan leading an alkalinization of local soil (Walker, 1962, Nye, 1981). However, this pH alkalinization is mainly micro-localized around the rhizoplan (Nye, 1981). Although fungi and soil bacteria implicated in the mycorhizosphere also influence the soil pH, the shift of pH measured could be not linked strictly to oxalotrophic activity. Therefore, the pH measurement protocol was not precise enough to

allow a significative correlation between soil pH and oxalotrophic community presence at the lowest horizon of the profile.

The alkaline pH (8.3) on the very top of the profile A is most probably related to the litter oxalotrophic activity as discussed before. The decrease pH values after ten first centimeters depth are also related to a decrease bacterial activity. Compared to profile C, the soil under the oxalogenic influence shows higher biological activity on the top and middle depths, thus pH measurements correlate significantly with qPCR measurments of oxalotrophic communities (r^2= 0.95 for India and 0.41 for Cameroon). The pH at the end of the profile (100 and 65 cm depth at Indian and Cameroon soils, respectively in profile A) is most likely related to small roots and rootlets observed in field (Fig. 1) rather to the oxalotrophic activity as discussed before, since at those depth points no oxalotrophic bacteria was detected taken into account that the limit of detection qPCR for *frc* gene is 10^2 copies per gram of soil. Therefore, the weak increase of the pH in the bottom of the profile could be related to root leaching processes.

iv. Comparison with other studied sites

Fields observations are well correlated with soil samples analysis. In this studied soil, was not able to accumulate secondary carbonates like reported under the tree *Milicia excelsa* in Ivory Cost (Cailleau, et al., 2005). However, this fact can easily be explained by the climatic differences between those sites specially with raining and bedrock lixiviation, since more than 1000 mm of rainfall, mainly concentrated on four month (June to September), most of the carbonates in surface are lixiviated (Cao, et al., 2001). Even though the presence of oxalate has not been assessed by direct measurement, the carbonates measurements as well as the microbiological analysis give evidences that the tree *Terminalia bellirica* is an oxalate producing tree with potential use in agrophorestal and ecological service programs.

At each region, environmental stress factors, such as extreme temperatures, and microchanges in soil pH or TOC content or low nutrient availability, may select for a particular diversity of oxalotrophs found with major consequences for carbon cycling through the assessed OCP ecosystems in India and Cameroun.

e. Conclusion

This study compare two systems being under tropical monsoon climate conditions, which could provide similar adaptive response by oxalotrophic bacteria, and that is exactly what we have seen, regarding the correlations between biotic and abiotic treats of the OCP systems assessed in

(*Terminalia bellirica* and *Milicia excelsa*, from India and Cameroon). The biome-specific mechanisms by which changes in the microbial composition may affect soil carbon sequestration through the oxalate pathway assessed in India and Cameroon are steel poorly described, nonetheless this investigation has shown that there is a positive correlations between key factors related with the activation of the oxalate-carbonate pathway. The presence of oxalotrophic bacterial communities, the shift of soil pH_{H2O} through alkalinization, and carbonate precipitation have been shown to be important conditions to characterize and compare the potential carbon sink pathway in the forests assessed. Futur research should be focus in studies *in situ* of plant-soil-bacteria measurements in which the ratio $CO_2/CaCO_3$ due to oxalotrophic activity would be quantified to put in evidence the real budget of carbon storage by the use of the oxalate-carbonate pathway.

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Vertical distribution of oxalotrophic bacteria in profile A carried out in India and Bolivia.

Interestingly the similar pattern of vertical distribution observed in both OCP systems at profile A fit very well with colony form units measurements carried out with the same soil samples. In addition, the DGGe profiles carried out using those soil samples shows that at each time a peak of increasing frc o UFC communities is observed, major diversity on the lanes is obtained (Figure 2).

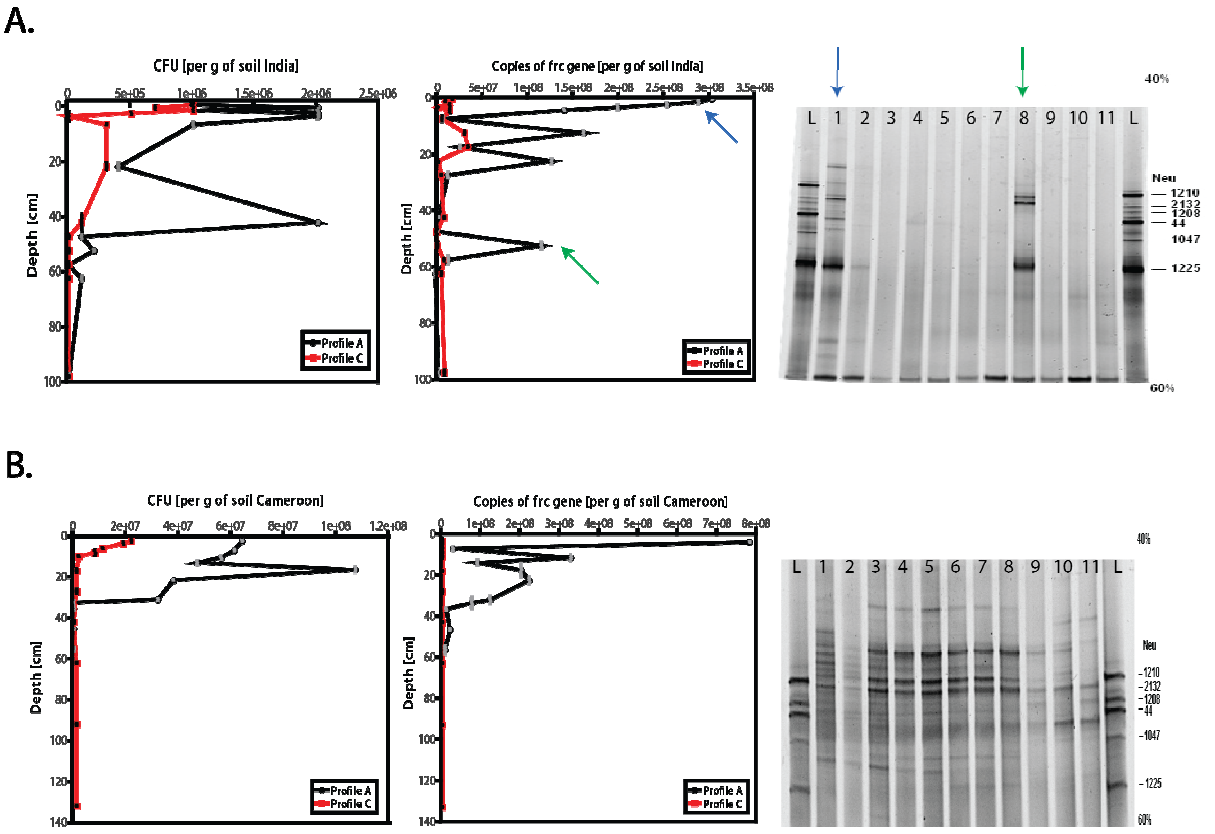

Figure 2. Colony-forming units (CFUs) counting, qPCR quantification of *frc* communities of Profiles A and C; and DGGE profile of the soil samples from profile A carried out in **A.** India, and **B.** Cameroon. Profile A (positive, in black) performed near to the trunk of an oxalogenic tree. Profile C (control; in red) corresponds to soil collected at 14 m from the tree trunk. Conventions: DGGE lines in both cases represent the depth in which soil samples were taken from profile A. DGGE lines for India: 1 = 0.5; 2 = 1.5; 3 = 2.3; 4 = 3.5; 5 = 4.5; 6 = 22.5; 7 = 42.5; 8 = 52.5; 9 = 62.5; 10 = 72.5; 11 = 97.5 cm deep. DGGE lines for Cameroon: 1 = 3; 2= 7; 3 = 11; 4 = 13; 5 = 17; 6 = 22; 7 = 31; 8 = 33; 9 = 36; 10 = 46; 11 = 56 cm deep. L = Ladder. The ladder for both Indian and Cameroon DGGE profiles was composed by a composite of *frc* amplificons of the oxalotrophic model strains: Neu 1210 = *Ancylobacter polymorphus*; Neu 2132 = *Alcaligenes paradoxus*; Neu 1208 = *Azospirillum brasilense*; Neu 44 = *Methylobacterium extorquens*; Neu 1047 = *Cupriavidus oxalaticus*; and Neu 1225 = *Streptomyces violaceoruber*. Most interesting changes in DGGE profile from India indicated with arrows (Blue = 0,5 cm and Green = 52.5 cm deep).

The structure of oxalotrophic community (in this case studied by UFCs and qPCRs of the gene *frc*), and the composition of the community (using the same *frc* amplicons to perform DGGEs profiles) are shown in figure 2. In both cases, the number of oxalotrophic bacteria obtained by qPCR was over two to three orders of magnitude higher than those obtained by the culture-dependent method. This result shows clearly the effect of the enrichment bias largely known in microbial ecology (Dunbar*, et al.*, 1997).

In general the comparison of the results obtained for the profiles under an oxalogenic tree and those of the control soil for all three sites showed that abundances of oxalotrophic bacteria was strongly stimulated by the presence of the tree at each site studied. In all the cases a difference of several orders of magnitude was observed as well, when comparing samples from equivalent depths between the profiles A and C.

DGGE profile of Indian soil samples revealed a greater diversity of oxalotrophic community at 0.5 and 52.5 cm deep (Figure 2A). This probably indicates that oxalate dispersion is a driven factor of oxalotrophic abundance in deep. The two depths, 0.5 and 52.5 cm (lanes 1 and 8), correspond to regions in which a high number of *frc* copies were found by the qPCR ($3.1x10^8$ and 1.2×10^8, respectively) (Figure 2A, showed with the blue and green arrows). Both DGGE lines have shown a strong intensity of band (according with the densitometric curve by GelCompare II) similar to the electrophoretic mobility observed in the oxalotrophic model strain *Streptomyces violaceoruber*. The presence of oxalotrophic actinobacterial-like phylotypes at that 1 and 52 cm deep in India, has been supported with the culturable study (chapter 4, supplementary tables 1 and 2) with the presence of the strains *Streptomyces* sp and *Terrabacter* sp., isolated at 1 and 50 cm depth respectively, and previously discussed in this global section.

The DGGE profile from Cameroon (Figure 4B) has shown overall that the composition the of oxalotrophic community do not change considerably in function of depth. A less strong signal of the bands was observed in lanes 1 and 2 (corresponding to 3 and 7 cm depth, respectively), albeit with a higher diversity of oxalotrophs. A strong signal of the bands (confirmed by the peaks found in the densitometric courve on the DGGE analysis using the software GelCompare II) was observed in lanes three to eight (11 to 33 cm deep). Those strong bands could be related with the populations found in the qPCR (with two peaks at 10 and 30 cm deep), and CFUs observed at similar deep (20 cm deep). Nevertheless, this should be confirmed. Thus, in further studies, identification of excised bands of oxalotrophic populations corresponding to the hotspots observed in the qPCR and by those lines showing more diverse patterns of bands in

DGGE profile might increase greatly our understanding of the oxalotrophs cohabiting at that oxalate reservoirs *in situ*. Interestingly, the DGGE profile from Cameroon shows little signs of high-GC content (e.g. actinobacteria-like) phylotypes in comparison with the DGGE profile from India. This could be just an hypothesis, however, chapters 3 and 5 have confirmed that in culture and unculturable oxalotrophic fractions of the soil samples from Cameroon there is a heterogeneity of bacteria, and the proportion of actinobacteria is less than the proportion found at Indian soil samples from the oxalogenic tree *T. bellirica*.

Those last results point out that release of oxalate sources occurs in the rhizospheric zone of the pedological profiles, especially between 20 and 55 cm for India and Cameroon (Figure 2 this chapter). The release of oxalic acid, and precipitation of Caox due to the structure of root systems in soils, and fungal activity around those systems, should be study in further research. In addition, it is highlighted the activity observed at the litter level, in which saprophytic fungi and bacteria (actinobacterial most likely) allow the liberation of important amounts of oxalate and other compounds probably related with the biodisponibility of Caox in soil. Likewise, at 50 cm deep in both Indian and Cameroon profiles A it was observed in the field trips the presence of large roots systems. Probably at that sampling point constant exudation of oxalic acid as a consequence of defense or detoxification responses by the oxalogenic three contribute greatly to the biodisponibility of the substrate for the oxalotrophic community. Therefore, the presence of major oxalotrophic bacteria related with the structure and composition of the community, have been an interesting proxy of the oxalate dispersion across the soil profiles.

Perspectives: New lines of research in the OCP and applications of the results from this study

Evolution and origin of oxalotrophy

The evolution of oxalotrophy is a topic less understood in the oxalate-carbonate pathway. Since a high diversity of oxalotrophs was found in tropical soils in this study, oxalotrophy should represent a highly adaptive metabolic process evolved in terrestrial ecosystems. Several stochastical changes should drive some groups of terrestrial bacteria to prefer oxalate as sole cabon and/or energy source, as it has been demonstrated with other carbon sources in soil profiles (Kramer & Gleixner, 2008). One selective force in oxalotrophic diversity could be the aerobic - anaerobic transition at the Precambrian. Anaerobic oxalotrophy occurs in soil and has been reported less studied in comparison with the effort performed using aerobic oxalotrophs (Blackmore & Quayle, 1968, Sahin, 2003, Daniel*, et al.*, 2007). A question that could be made is if anaerobic oxalotrophs are implied in the oxalate-carbonate pathway. It is known that the origin of the metabolism of dicarboxilic acids constitute a small fraction of the polyphyletic evolution of metabolic types occuring at the precambrian (Schlegel, 1986, Fani & Fondi, 2009). Nevertheless, never before evolution of oxalotrophs has been studied. Therefore, some relevant questions remain in the origin and ecological role of oxalotrophy in terrestrial environments.

Use of oxalotrophic bacteria to develop biofertilizers

The oxalotrophic bacterial strains characterized in this study could be used for further studies to facilitate the production of biofertilizants, due principally to the fact that an important fraction of oxalogenic bacteria found so far in tropical soils corresponds to rhizospheric nitrogen fixers, mostly related with Rhizobia (symbiotic diazotrophic bacteria). That group might help to increase oxalogenic plant productivity (high levels of RUBISCO, which is involved in the production of oxalic acid), consequently increasing atmospheric CO_2 fixation *in situ* at high levels and at long-term. This would be especially important for tropical soils with elevate atmospheric CO_2 concentrations and poor N_2 fixation.

The link opened in this study through fungi-bacteria interactions (chapters 5 and 7) in the context of the OCP, is a key factor to regulate global warming. A schematic proxy using the "potential of synergy" to maximize the efficiency of that particular niche (involving both, bacteria and fungi) in the OCP studied is shown in figure 3.

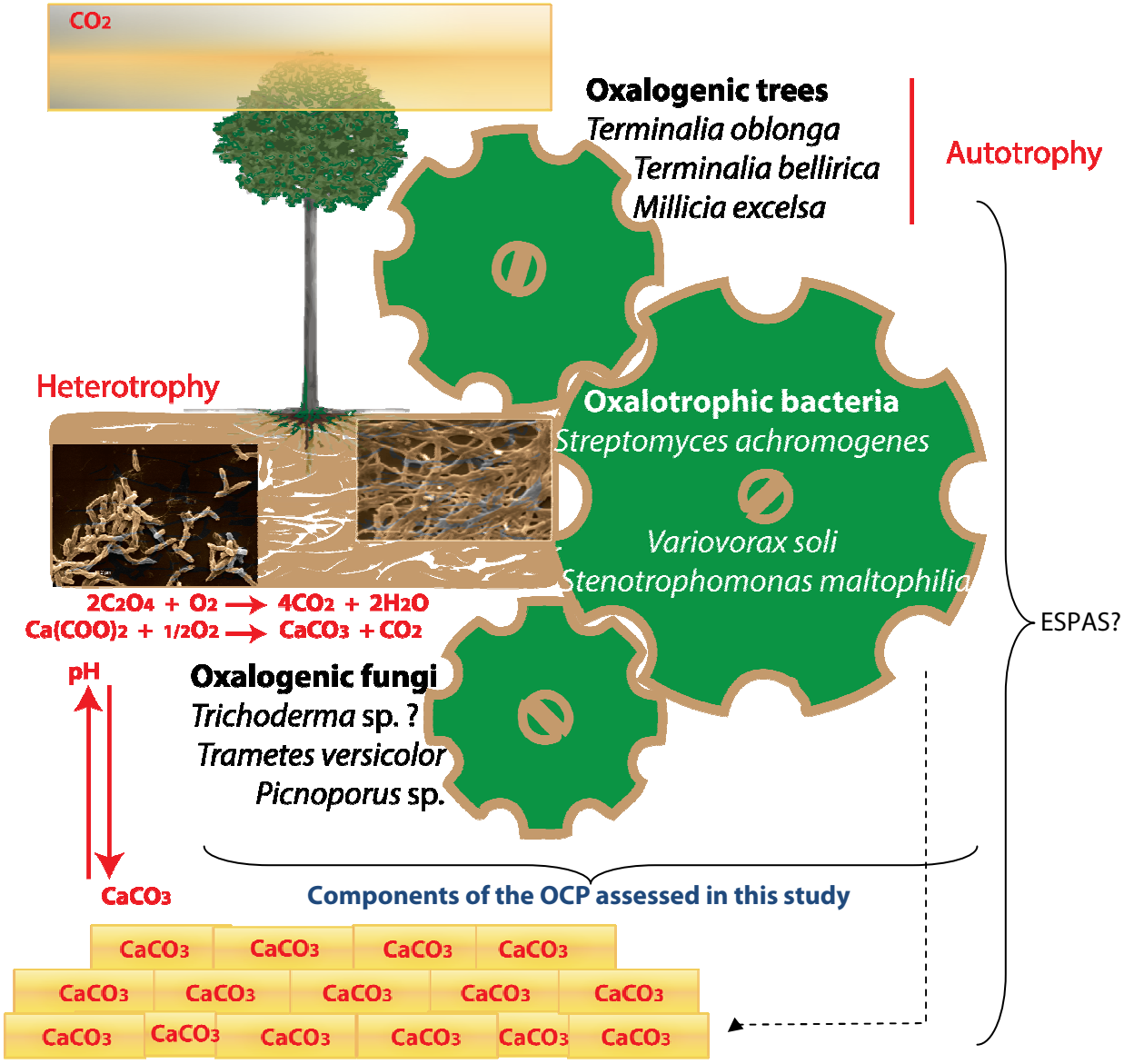

Figure 3. Sketch showing the link between oxalotrophic bacteria considered with high impact due efficiency of oxalate oxidation (measured by consumption rates and net-growth rates, and by kinetic curves of growth) over the oxalogenic tree in which they were isolated. The sketch shows the OCP as a gear composed by their biological components (oxalogenic plants and fungi, and oxalotrophic bacteria) and the geological function at long term.

The OCP systems investigated have been shown to be suitable for the proliferation of oxalotrophic bacteria, regarding the diversity assessed by different methods. Still the efficiency and capacity of each system in the main goal to tackle global warming caused by atmospheric CO_2 at a local scenario is a task to be resolved. Nevertheless, the contribution of this thesis in the aim of describing and quantifying potential microbiological forces as a major gear implied in the activation of an effective OCP (figure 3) has been relevant for tropical habitats.

The use of the OCPs systems studied in tropical forest must involve the estimulation of several environmental programs at global scale that include fertilization of tropical soils using oxalotrophic rizhospheric microbiota associated to oxalogenic plants such those assessed here, or achieving biomineralization of oxalate into carbonate as a CO_2 trap strategy. Consequently, environmental policies should take into account the oxalate-carbonate pathway as a long-term strategy, nonetheless durable to deal with global warming.

The purpose of this study was to illustrate how diverse and how efficient could be the metabolic potential within the bacterial world implied on the activation of the OCP mechanism in tropical forest from Bolivia, India and Cameroon. The perspectives of this work should include the use of that diversity and metabolic potential, to perform scaled-up systems, such as the experimental soil-plant-atmosphere (ESPAS) systems in the near future (Figure 4). This would allow measuring the effectiveness of the atmospheric CO_2 reduction into carbonate in a closed OCP system. For that purpose, new herbaceous oxalogenic plants should be taken into account, as well as the mycorrhizal effect over oxalotrophic bacterial dispersion and development in proportion with calcium oxalate reservoirs dispersed all around the complex matrix phyllosphere-rhizosphere. I believe that increasing our knowledge in that direction will give us an improvement about where, what, and when an OCP system could be exploited and, thus, to pass from a "potential carbon sink", into a major worldwide strategy to tackle high emission of atmospheric CO_2.

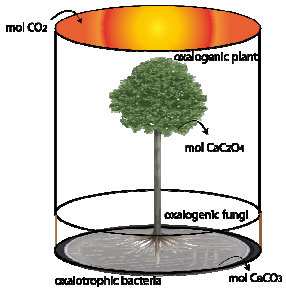

Figure 4. ESPAS systems that could be designed in the near future to assess real solutions to climate change using oxalotrophic bacteria through the oxalate carbonate pathway.

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Chapter 7. Other projects: Fungi, bacteria and soil pH: the oxalate–carbonate pathway as a model for metabolic interaction

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How to assess the biogeochemical interactions between soil pH, oxalogenic fungi and oxalotrophic bacteria occurring in the OCP?

Chapter summary

This chapter shows the first study performed at microcosm scale trying to simulate natural conditions in which physical factors (such as local soil pH), and biological interactions (such as those observed between oxalogenic fungi and oxalotrophic bacteria) are observed. This chapter highlights the importance of such biological interactions, affecting the physicochemical conditions in the sourounding nicht at the OCP.

Abstract

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.

a. Introduction

Oxalic acid ($H2C_2O_4$) 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).

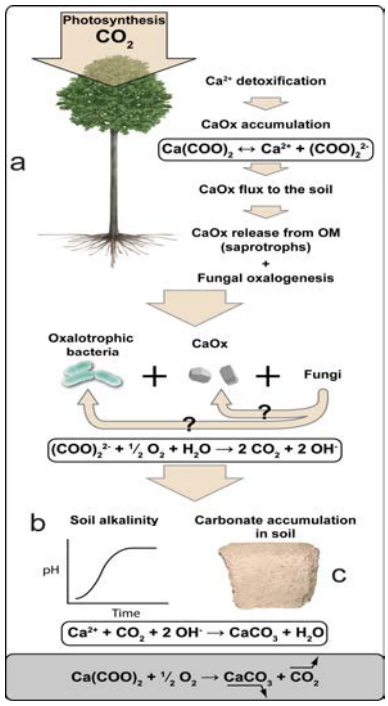


Fig. 1. Schematic representation of the oxalate–carbonate pathway showing the main biological players and the chemical reactions involved (modified from Aragno and Verrecchia, 2012). The unknown contribution of fungi as calcium oxalate producers and the role of their interaction with bacteria are indicated 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.

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 ($Ksp = 2.32 \times 10^{-9}$), 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 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 Gramnegative (*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.

b. Experimental procedures

i. 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 andAragno, 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 SchottTM 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 x 10^8 cells per ml were prepared separately, then mixed in equal amounts, and finally added to the microcosms to a final concentration of 10^7 cells per species and per gram of soil.

Table 1. Bacterial strains used for the inoculation of microcosms. Ref N° 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); TSA = Tryptone Soya Broth + 13 g•L^{-1} Agar; SDS = Sodium dodecyl sulphate. Last column gives the value of the melting temperature of *frc* gene qPCR amplicons. NA= not applicable.

Ref N°	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

ii. 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 cm3 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.

iii. 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 (pHKCl 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.

iv. 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 mm filter (X 50 Analypore PES 33 mm 0.2 mm, 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.

v. 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 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 (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.

vi. Soil pH measurements

Soil was dried overnight at 105 °C. A volume of 1.25 mL of deionised water was added to 0.525g (+/- 0.025g) of soil, placed on a rotary shaker for 2 hours, and centrifuged at 16000 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 to 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 T-test. The samples compared were considered as having a similar distribution.

vii. DNA extraction

DNA was extracted using the FastDNA® Spin Kit for Soil (Qbiogene, Inc. 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 analyzed as individual replicates. For the second set, DNA was extracted from three individual microcosm replicates.

viii. 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 analyzed 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 analyzed in a single qPCR run in order to minimize experimental error (Smith & Osborn, 2009). For quantification three independent standards series with 10^3 to 10^7 gene copies /µL were included. Standards were prepared with

known amounts of plasmid DNA containing a fragment of the target gene. The significance of the difference between values was assessed as mentioned for the pH measurements.

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

Gene	Primers	Primer conc.	DNA conc.	Program	Reference
frc	F: 5'-CTSTAYTTCACSATGCTSAAC-3' R: 5'-GDSAAGCCCATVCGRTC-3'	1.25 µM	1.6-4 ng/µL	40 cycles of 30s-95°C; 60s-56°C; 30s-72°C	Khammar et al., 2009
16S rRNA gene	F: 5'-ACTCCTACGGGAGGCAGCAG-3' R: 5'-ATTACCGCGGCTGCTGG-3'	0.3 µM	1.6-4 ng/µL	35 cycles of 10s-95°C; 15s-55°C; 20s-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 µM	1.6-4 ng/µL	35 cycles of 10s-95°C; 15s-55°C; 20s-72°C	Lopez-Garcia et al 2001* Fierer et al., 2005

*modified by using primer in reversed complement orientation

ix. Quantification of ergosterol

The method was adapted from Young (1995) and Larsen *et al.* (2004). Briefly, 4 mL of methanol and 1 mL of 2N NaOH were added to 2 g of lyophilised 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 2N HCl was added. The tubes were centrifuged at 400 rpm for 2 min and the supernatant transferred to new tubes. Two mL of pentane were added for ergosterol extration. 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 were 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 minutes and was detected at 280 nm. Ergosterol (Sigma) stock solution and standards were prepared in methanol.

c. Results

i. 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, Cailleau*, et al.*, 2005). We evaluated the influence of amendments with bacteria, fungi, and oxalate on changes in soil pH in seven different microcosms (Figure 2).

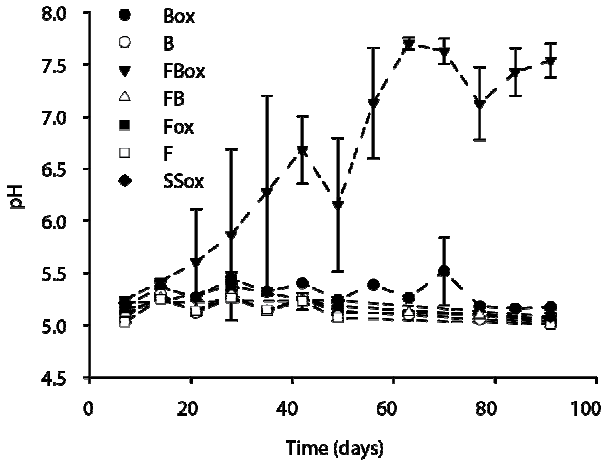
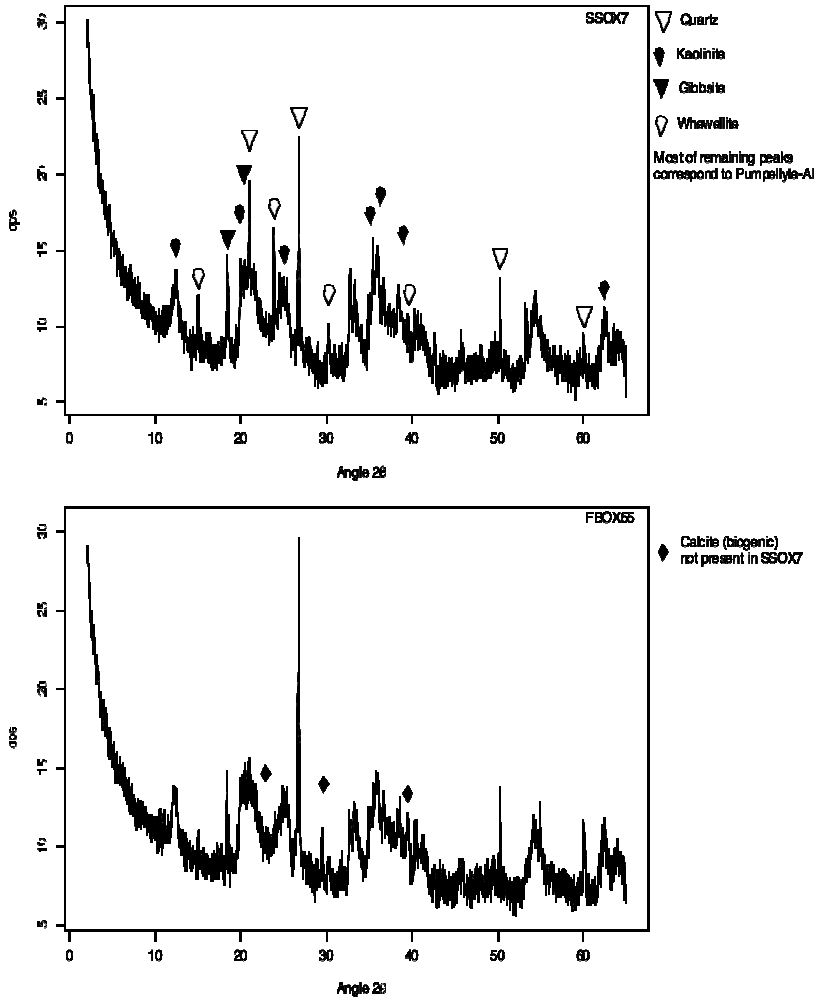


Figure 2. Evolution of pH in microcosms created with a sterile soil and inoculated with allochthonous microbial community. The different treatments were: inoculation with bacteria and Ca-oxalate amendment (Box), inoculation with bacteria, without Ca-oxalate amendment (B), inoculation with bacteria and 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.

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 (Supplementary Figure 1).



Supplementary Figure 1. XRD analysis showing the precipitation of calcite in the FBox treatment (down panel) compared to the sterile soil in presence of oxalate (Ssox treatment; upper panel). Mineralogical composition determinations were performed using a Scintag diffractometer. X-ray diffractogram were analyzed 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.

ii. 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 (Figure 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*: $3.7x10^4$ to $2.8x10^8$; 16S rRNA gene: $2.3x10^6$ to $3.8x10^9$). 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 (Figure 3). In the bacteria-free microcosms, *frc* gene concentration was below the detection limit (data not shown).

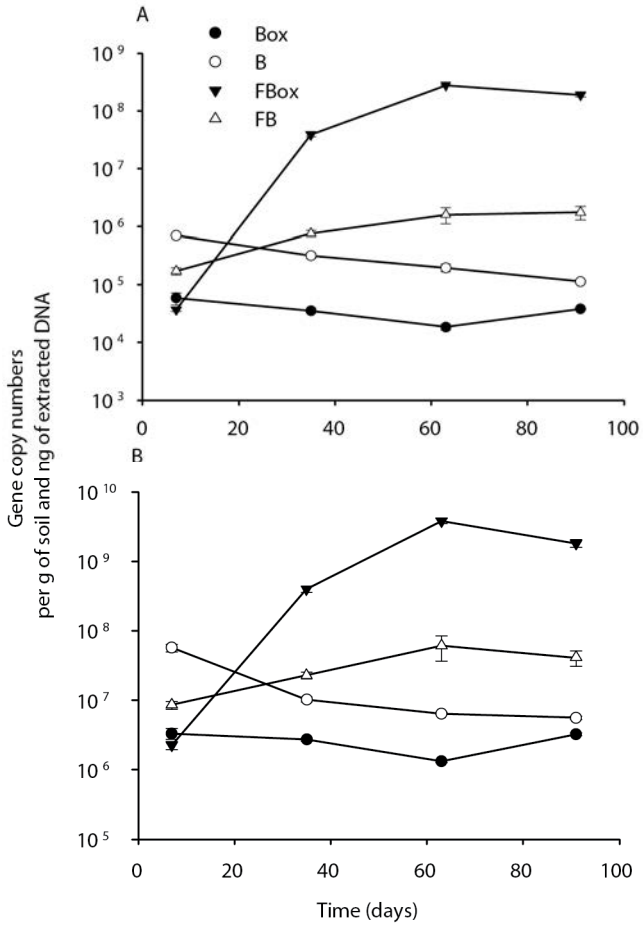
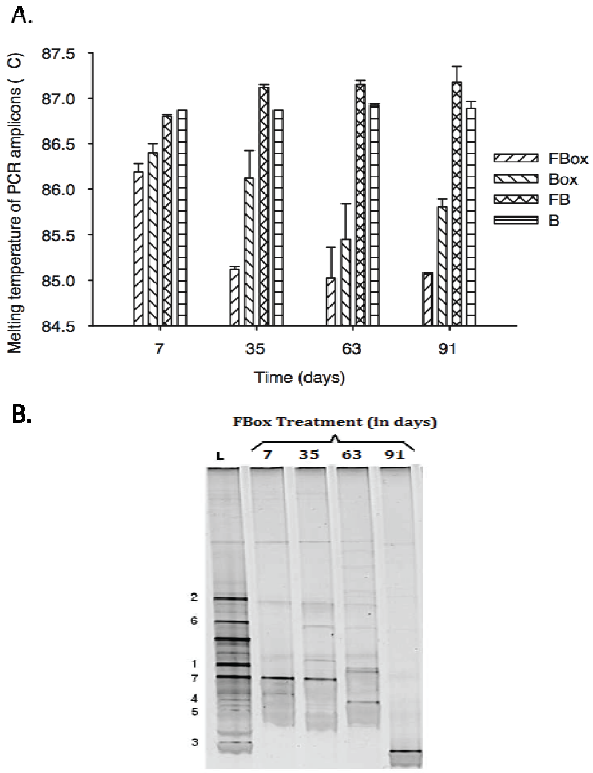


Figure 3. Quantification of *frc* gene (A) and 16S rRNA gene (B) copy numbers by qPCR in microcosms with sterile soil and inoculated with allochthonous microbial community. Mean values and standard deviations of replicate quantification (n=3) for three separate DNA extracts are given. For abbreviations see caption of Figure 1.

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 (Supplementary Figure 2A). 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). By 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 (Supplementary Figure 2B). 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 *Pandorea* sp., were detected only at two of the three time points. In the final point (91 days), a not easily identifiable band was observed.



Supplementary Figure 2. 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 program was used for nested PCR. A DCode System (BioRad) 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 0.5X TAE) during 5h at 150V 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).

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 (Figure 4). However, in treatments containing fungi and bacteria (FBox and FB), ergosterol concentrations fluctuated over time compared to the treatments with fungi alone (Fox and F).

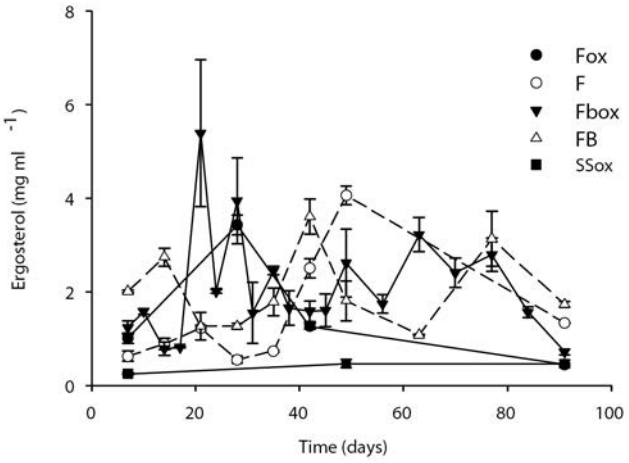


Figure 4. Quantification of ergosterol in microcosms with sterile soil and inoculated with allochthonous microbial community. Mean values (± standard deviations) of the quantification for three separate analyses. For abbreviations see caption of Figure 1.

iii. 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 (Figure 5). In microcosms with the native microbial community, the pH rose by about one unit within two weeks after calcium oxalate addition but remained unchanged in the treatments with the biocide mix (SSox analogue; Figure 5). Furthermore, maximum pH (pH 8) was reached in less than ten days as compared to eight weeks in the first experiment (Figure 2). A shift in soil pH was observed under two experimental conditions: no biocide treatment (FBox analogue) and cycloheximide-treated soil (Box analogue).

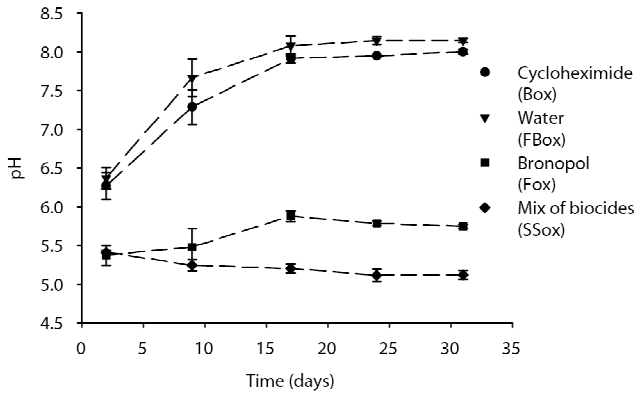


Figure 5. Evolution of pH in soil microcosms with native microflora and treated with specific biocides. Values are means (± standard deviations) of measurements performed on three separate microcosms. For abbreviations in brackets see caption of Figure 1.

iv. Effect of biocides on bacteria and fungi

The results from the microcosms with native microbial community (FBox analogue, Figure 5) confirmed those of the first microcosm series (Figure 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 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 (Figure 6). The bacterial and fungal abundances in this treatment were at least three orders of magnitude higher than in the control (Sox 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 to 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.

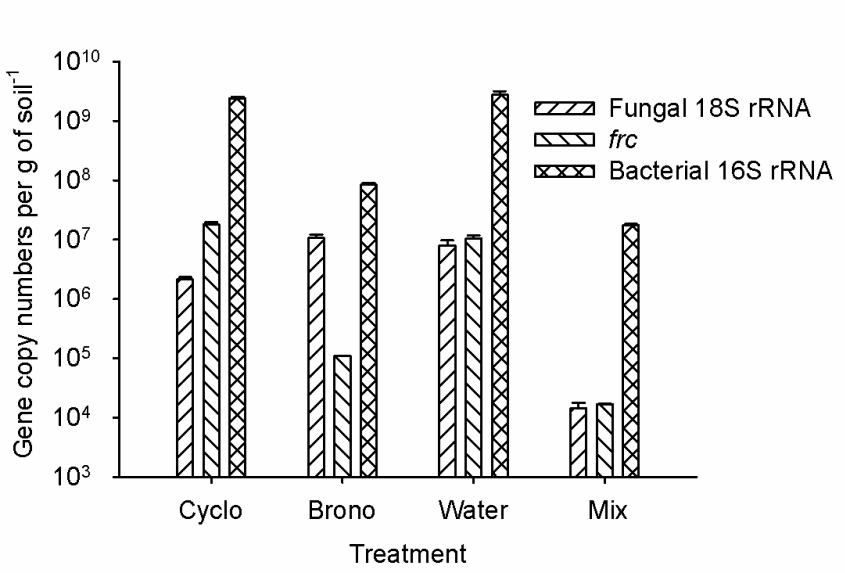


Figure 6. Quantification of *frc*, 16S rRNA gene and 18S rRNA gene copies at the end of the experiment performed in soils with native microflora and treated with biocides. Values are means (± standard deviations) of quantifications performed with DNA from three replicate microcosms.

d. Discussion

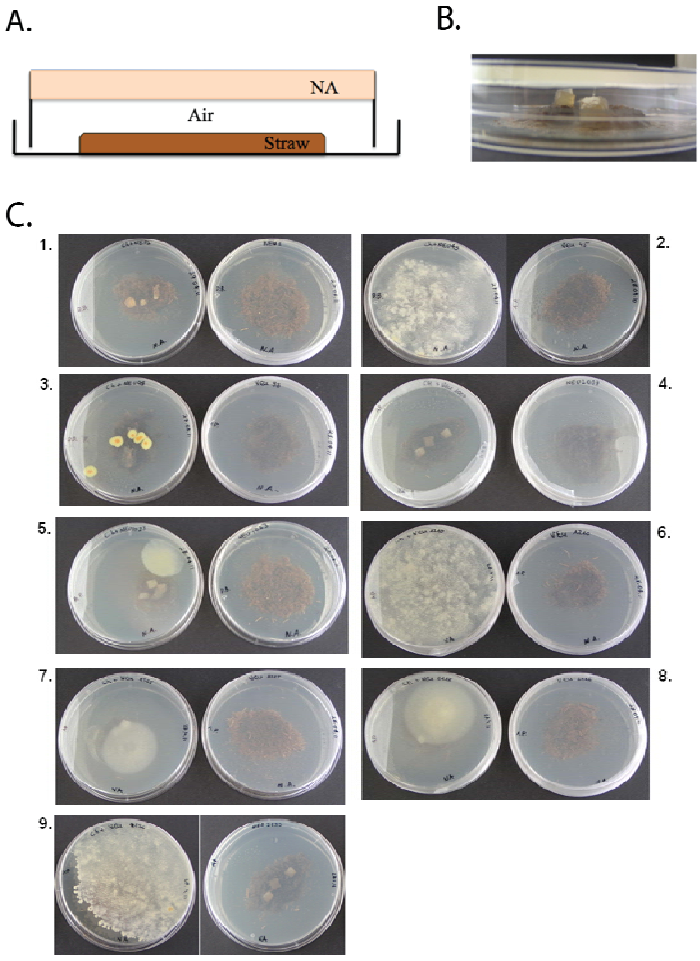
Experiments carried out with either an artificial or a native microbial community showed consistently that the simultaneous presence of bacteria, fungi, and oxalate is essential to induce the alkalinisation of the soil pH by up to 2.5 units and the precipitation of calcite, which are two keys effects 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 (Aragno & Verrecchia, 2012).

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 & 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 colonisation 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 *Pandorea* sp., *A. 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 (Supplementary Figure 3).



Supplementary Figure 3. 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, developing later on the target medium. **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*.

This mechanical transport facilitate the access of bacteria to nutrients, provisioning growth factors and organic compounds (i.e. fungal exudates), as well modifying microenvironmental conditions, and therefore, representing 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 & 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 & 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 *P. cinnabarinus*, suggesting that this protective role of fungi may have played a role in the microcosms as well (Supplementary Table 1)

Supplementary Table 1. 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 mediums 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 at 30°C in the dark. Growth was checked every 24 hours 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.

Strain	Medium	Growth pH 7	Growth pH 5	Growth pH 5 + fungus
Pandoraea sp.	NA	+	+	N.D.
Oxalicibacterium flavum T	ST1	+	-	-
Ancylobacter polymorphus	NA	+	-	+ (6 d)
Streptomyces violaceoruber	NA	+	-	-
Cupriavidus necator	NA	+	-	+ (5 d)
Variovorax paradoxus	NA	+	-	-

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 & 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 and cannot be confirmed.

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 demonstrated 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.

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Annexes

Poster and Oral presentations
Scientific CV Daniel Bravo

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Poster presentations

Abstract submitted for a Poster Presentation in the 69th Swiss Society for Microbiology (SSM) Congress, Zürich, Switzerland, 24- 25 June 2010

Characterization of new oxalotrophic bacterial strains from Bolivia and India

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Oxalic acid and calcium oxalate are widely distributed among plants, animals, fungi, and bacteria. Oxalic acid often accumulates as a metabolic product in plant cells, most commonly as the insoluble salt "calcium oxalate". Despite its relative insolubility and chemical stability, the accumulation of metal oxalates has rarely been observed in the geological record. This supposes a microbiologically mediated process as the main oxalate sink in natural environments. Oxalotrophic bacteria are able to use oxalate as a source of carbon and energy. These bacteria do not constitute a phylogenetic group but rather a functional group that consists of diverse taxa of microorganisms. Recently, oxalotrophic bacteria have been singled out as one of the main biological components of a novel and underestimated long-term carbon sequestration mechanism: the oxalate-carbonate pathway. In this pathway, oxalotrophic bacteria use the oxalate produced by plants and fungi, leading to the production of carbonate that can precipitate in the form of calcium carbonate in acidic tropical soils developed on carbonate-free parental material. In this study, new strains of oxalotrophic bacteria were identified and enriched from two tropical soils in which the oxalate-carbonate pathway has been detected. Enrichments in solid and liquid media were prepared using soils collected under oxalogenic trees Verdolago (*Terminalia oblonga*; Bolivia) and Bahera (*Terminalia bellirica*; India), as well as from soils that were not under the influence of the trees (control soils). Evidence of oxalotrophy was obtained through the solubilization of Ca-oxalate as sole carbon source in mineral medium. More than a hundred new strains of oxalotrophic bacteria were isolated. The kinetics of oxalate consumption in the strains is currently studied using microcalorimetric methods. The strains will be identified using the 16S rRNA gene sequence. The presence of the *frc* gene, a functional marker for oxalotrophy, will also be tested in all the new strains.

Abstract submitted for a Poster Presentation in the 4th Swiss Microbial Ecology (SME) Meeting February 2 - 4, 2011 Engelberg, Switzerland.

Use Of Isothermal Microcalorimetry Assay (MCA) To Characterize Microbial Oxalotrophic Activity

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Abstract

Isothermal microcalorimetry (IMC) assay has been used in the past to monitor specific metabolic activities measured in terms of the heat production rate of living cellular systems. With respect to microbes, applications include biotechnological, medical and agricultural research. However, only few studies have focused on ecological studies of soil microorganisms. In our laboratory, we are interested in characterizing oxalotrophy, an important and unusual bacterial process occurring in natural systems. Oxalotrophic bacteria have been normally detected by traditional microbiological techniques, and more recently, by molecular biology. This metabolic activity is especially relevant in soils in which the oxalate-carbonate pathway has been detected. The oxalate-carbonate pathway is a process that leads to carbon sequestration by the assimilation of atmospheric CO_2 and subsequent precipitation of calcite, linking the action of plant, fungi and bacteria. In this biogeochemical cycle the role of soil bacteria is essential, because the consumption of oxalate releases carbonate ions and calcium ions. These 2 ions are consequently transformed abiotically into calcium carbonate (calcite). The exergonic reactions which allow bacteria to obtain energy from oxalate as substrate can be measured by IMC. A greater challenge is to measure oxalotrophic activity directly in soil.

To evaluate the potential of IMC to measure oxalotrophic activity, six reference strains and two isolates from Bolivian tropical soils were inoculated in solid angle media with potassium oxalate as sole carbon source. The results obtained showed that *Cupriavidus oxalaticus* NEU1047, *Cupriavidus necator* NEU2073, and *Streptomyces violaceoruber* NEU1225 developed the highest activity in microwatts (91, 40, 55 respectively) and highest μmax kinetic parameter (0.264, 0.185, and 0.199 respectively). These results were confirmed by growth kinetics in liquid media showing the highest correlation between growth and oxalate degradation. The same strains were selected to evaluate the incidence of oxalate source (i.e., insoluble metal oxalates) in their metabolic activity. Ca, Mg, Cu, and Fe Oxalate salts were tested because their environmental relevance in the natural process. The highest response of heat flow was obtained with Ca oxalate source in *C. oxalaticus* NEU1047. To test whether the same approach could be used to measure oxalotrophic activity *in situ*, similar experiments were carried out in a model soil. In soil, the results were Z. The directly correlation between heat flow and curves obtained by HPLC chromatograms suggest that IMC analysis is a powerful method to monitor bacteria oxalotrophic activity.

Abstract submitted for a Poster Presentation in the 4th Congress of European Microbiologists FEMS 2011, Geneva, Switzerland June 26 - 30, 2011.

Use of an Isothermal Microcalorimetry Assay to Characterize Microbial Oxalotrophic Activity

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Isothermal microcalorimetry (IMC) has been used in the past to monitor metabolic activities in living systems. A few studies have used it on ecological research. In this study, IMC was used to monitor oxalotrophic activity, a wide spread bacterial metabolism found in the environment, and particularly in soils. Six model strains inoculated in solid angle media with K-oxalate as the sole carbon source. *Cupriavidus oxalaticus*, *Cupriavidus necator*, and *Streptomyces violaceoruber* presented the highest activity (91, 40, and 55 microwatts, respectively) and a maximum growth rate ($\mu max \cdot h^{-1}$) of 0.264, 0.185, and 0.199, respectively, among the strains tested. These three strains were selected to test the incidence of different oxalate sources (Ca, Cu, and Fe-oxalate salts) in the metabolic activity. The highest activity was obtained in Ca-oxalate for *C. oxalaticus*. Similar experiments were carried out with a model soil to test if this approach can be used to measure oxalotrophic activity in field samples. Although measuring oxalotrophic activity in a soil was challenging, there was a clear effect of the amendment with oxalate on the metabolic activity measured in soil. The correlation between heat flow and growth suggests that IMC analysis is a powerful method to monitor bacterial oxalotrophic activity.

Abstract submitted for a Poster Presentation in the 10th Swiss Geoscience Meeting, Bern, Switzerland October, 2012

The oxalate-carbonate pathway: at the interface between biology and geology

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The formation of calcite in otherwise carbonate-free acidic soils through the biological degradation of oxalate is a mechanism termed oxalate-carbonate pathway. This pathway lies at the interface between biological and geological systems and constitutes an important, although underestimated, soil mineral carbon sink. In this case, atmospheric CO_2 is fixed by the photosynthetic activity of oxalogenic plants, which is partly destined to the production of oxalate used for the chelation of metals, and particularly, calcium. Fungi are also able to produce oxalate to cope with elevated concentrations of metals. In spite of its abundance as a substrate, oxalate is a very stable organic anion that can be metabolized only by a group of bacteria that use it as carbon and energy sources. These bacteria close the biological cycle by degrading calcium oxalate, releasing Ca^{2+} and inducing a change in local soil pH. If parameters are favourable, the geological part of the pathway begins, because this change in pH will indirectly lead to the precipitation of secondary calcium carbonate (calcite) in unexpected geological conditions. Due to the initial acidic soil conditions, and the absence of geological carbonate in the basement, it is unexpected to find C in the form of calcite. The activity of the oxalate-carbonate pathway has now been demonstrated in several places around the world, suggesting that its importance can be even greater than expected. In addition, new roles for each of the biological players of the pathway have been revealed recently forcing us to reconsider a global biogeochemical model for oxalate cycling (Martin et al., in press).

Abstract submitted for a Poster Presentation in the 5th Congress of European Microbiologists FEMS 2013, Leipzig, Germany Jully 21 - 25, 2013. Abstract topic: Environmental microbiology

ISOLATION AND CHARACTERIZATION OF NEW OXALOTROPHIC BACTERIA FROM TROPICAL SOILS

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The oxalate-carbonate pathway (OCP) implies the conversion of atmospheric CO_2 into calcium carbonate. This process takes importance in tropical soils due to high yielding of calcite precipitation. At the biological level, the oxalotrophic bacteria are determinant to activate the pathway by oxidation of calcium oxalate (Ca-oxa). Some studies have been carried out to assess the diversity of oxalotrophic bacteria independently of the OCP; however the diversity of oxalotrophs as part of the OCP at global scale is unknown. Therefore this research is focus on the characterization of new oxalotrophic bacteria in three tropical soils, using both molecular and analytical approaches. Ninety seven oxalotrophic strains have been isolated and identified by sequencing of 16S rRNA gene and characterized as oxalotrophic bacteria by plating in Schlegel medium with Ca-oxa. Thirty strains were obtained in soil collected in Bolivia (Tree *Terminalia oblonga*), 27 strains were isolated in soil collected in India (Tree *Terminalia bellirica*), and 40 strains were isolated in soil collected in Cameroon (Tree *Millicia excelsa*). Ten bacterial strains were chosen due to degradation halos observed in plates with Ca-oxa. Four strains amplifying the *frc* gene shows that an alternate catabolic way may occur when oxalate is oxidize in *frc* negative strains. Kinetic curves in batch cultures and microcalorimetric thermograms shows that the strains C18 *Variovorax soli*, A9 *Streptomyces achromogenes* and C33 *Stenotrophomonas maltophilia* have highest oxalate consumption rates with 0.24, 0.17 and 0.15 $\mu M.h^{-1}$ respectively. Thus, the role of *Variovorax, Streptomyces* and *Stenotrophomonas* species is discussed in the OCP.

Oral presentations

Abstract submitted for ***Oral Presentation*** *in the Doctoral Meeting as part of the Doctoral program in organismal Biology UNINE, May 17th, 2011in Neuchâtel, Switzerland.*

Isothermal Microcalorimetry As a new tool to quantify microbial oxalotrophic activity

Daniel Bravo[1], Olivier Braissant [2], Anna Solokhina[2], Martin Clerc[1], Alma U. Daniels[2], Eric Verrecchia[3], Pilar Junier[1]

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Abstract

Isothermal microcalorimetry (IMC) has been used in the past to monitor specific metabolic activities measured as heat production rate in living systems. IMC have been used in soil research particularly in three different ways: (i) characterization of soil microbial activity, (ii) monitoring the toxicity - biodegradation of soil organic pollutants and (iii) the risk evaluation of metals and metalloids. However, in soil microbial activity few studies have focused on IMC use for characterize oxalotrophic activity. We are interested in characterizing oxalotrophy, because it is an unusual bacterial process occurring in natural systems. This metabolic activity is especially relevant in soils in which the oxalate-carbonate pathway has been detected. In this biogeochemical pathway that links the action of plants, fungi and bacteria, the consumption of oxalate by soil bacteria is essential, because it releases carbonate and calcium ions. These two can be transformed abiotically into calcium carbonate (calcite) leading to the formation of deposits such as needle fiber calcite, or calcite-cemented sandstone. Enrichment cultures have been normally used to detect oxalotrophic bacteria and more recently, a culture-independent approach has also been developed. However, the exothermic reactions, that allow bacteria to obtain energy from oxalate as substrate, can be measured by IMC and thus, IMC has a potential to be used for the detection of such metabolic activity in field samples with a high sensitivity.

Abstract submitted for ***Oral Presentation*** *in the 8th International Symposium of Subsurface Microbiology ISSM 2011, September 11 - 16, 2011in Garmisch-Partenkirchen, Deutschland.*

Use of An Isothermal Microcalorimetry Assay To Characterize Microbial Oxalotrophic Activity

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Abstract

Isothermal microcalorimetry (IMC) has been used in the past to monitor specific metabolic activities measured as heat production rate in living systems. IMC have been used in soil research particularly in three different ways: (i) characterization of soil microbial activity, (ii) monitoring the toxicity - biodegradation of soil organic pollutants and (iii) the risk evaluation of metals and metalloids. However, in soil microbial activity few studies have focused on IMC use for characterize oxalotrophic activity. We are interested in characterizing oxalotrophy, because it is an unusual bacterial process occurring in natural systems. This metabolic activity is especially relevant in soils in which the oxalate-carbonate pathway has been detected. In this biogeochemical pathway that links the action of plants, fungi and bacteria, the consumption of oxalate by soil bacteria is essential, because it releases carbonate and calcium ions. These two can be transformed abiotically into calcium carbonate (calcite) leading to the formation of deposits such as needle fiber calcite, or calcite-cemented sandstone. Enrichment cultures have been normally used to detect oxalotrophic bacteria and more recently, a culture-independent approach has also been developed. However, the exothermic reactions, that allow bacteria to obtain energy from oxalate as substrate, can be measured by IMC and thus, IMC has a potential to be used for the detection of such metabolic activity in field samples with a high sensitivity.

Five reference strains and two isolates from a tropical soil have been inoculated in solid angle media with K-oxalate as sole carbon source in order to evaluate the potential of IMC to measure oxalotrophic activity. The obtained results show that *Cupriavidus oxalaticus*, *Cupriavidus necator*, and *Streptomyces violaceoruber* present the highest activity (91, 40, and 55 microwatts, respectively) and growth ($\mu max \cdot h^{-1}$) = 0.264, 0.185, and 0.199, respectively). These results are confirmed by kinetics in liquid media showing a correlation between growth and oxalate degradation. The incidence of the oxalate source in the metabolic activity is evaluated as well. Ca, Mg, Cu, and Fe-oxalate salts have been tested because of their environmental relevance. The highest activity is obtained for *C. oxalaticus* in Ca-oxalate. Similar experiments have been carried out in a model soil in order to test whether the same approach can be used to measure oxalotrophic activity in field samples. Although measuring oxalotrophic activity in a soil is a greater challenge, there is a clear effect on metabolic activity in the presence or absence of added oxalate. The correlation between heat flow and growth curves suggests that IMC analysis is a powerful method to monitor bacterial oxalotrophic activity.

Abstract submitted for ***Oral Presentation*** *in the 5th Swiss Microbial Ecology (SME) Meeting February 4 - 6, 2013, Löwenberg, Murten, Switzerland.*

Identification of active oxalotrophic bacteria by BrdU labeled-DNA and its importance in the oxalate-carbonate pathway in natural habitats

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[§] These authors have contributed equally to this study.

Abstract

The oxalate-carbonate pathway is probably one of the most important and underestimated potential carbon sink in terrestrial environments. To assess such biogeochemical process in tropical habitats several elements have to be taken into consideration including, the geology of the site, the presence of oxalogenic trees and fungi, and the presence of oxalotrophic bacteria. New insights into the diversity of culturable oxalotrophic bacteria been obtained recently for three sites. However studies focused in diversity using just the culturable fraction of the oxalotrophic bacterial communities are biased as this represents just a small fraction of the total diversity *in situ*. In addition, the isolation or even the molecular characterization of oxalotrophic bacteria, is not necessarily informative regarding the active players into the oxalate-carbonate pathway. Therefore the aim of this study was to assess the diversity of the active oxalotrophic bacterial communities using the BrdU DNA labeling technique in a microcosm system. The soil employed was collected in an oxalogenic system aside an Iroko tree in Cameroon. The microcosms treatments consisted of the addition of calcium oxalate (0.5, 1 and 4% w/w respectively). Those were compared to an untreated control. After 12 days of incubation a maximal pH value of 7.7 was detected in the treated microcosms (initial pH of XX) due to the oxalotrophic metabolism. At this time point, a DGGE profile was performed using both BrdU labeled and unlabeled soil DNA. The *frc* gene was used as molecular marker for oxalotrophy. Populations of Actinobacteria composed by the genera *Streptomyces*, *Kribbella* and *Nocardiopsis* were found as main group of the active oxalotrophic bacterial communities (48% of 65 sequenced DGGE bands). Those were followed by gamma and beta - Proteobacteria representing 19 and 13% of the active community, respectively. Besides, no difference in community composition was observed when different concentrations of calcium oxalate were amended to the soil. This study highlights the relevance of Actinobacteria as the main members of the active bacterial community in the oxalate-carbonate pathway.

Curriculum Vitae

Personal information

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Nationality	Colombian	Marital status:	Single
Date of birth	12.08.1981	Children:	None

Employment

Dates	January 2010 to 2013
Position held	Doctoral Assistant in Microbiology, University of Neuchâtel, Switzerland
Main activities and responsibilities	Study of oxalotrophic bacteria in tropical soils, at the laboratory of Microbiology, University of Neuchatel. My duties include teaching and research.
Dates	July 2008 to October 2009
Position held	Half-time Professor in Microbiology, University of Nariño, Colombia
Main activities and responsibilities	Teaching General Microbiology. My duties included research and collaboration in projects related with Microbial ecology in soils ecosystems from Andean region of Nariño, Colombia.

Education

Dates	2010-2013
Title awarded	PhD in Microbiology
Principal subject	Assessing the diversity and metabolism of oxalotrophic bacteria in tropical habitats
	University of Neuchâtel, Switzerland
Dates	2003-2008
Title awarded	Biologist (equivalent to a Master)
Principal subject	Synthesis of Polyhydroxyalkanoates in diazotrophic bacteria isolated from nodules of sylvester legume plants (*Faboideae*) in Colombian Andes
	University of Nariño, Colombia

Teaching record

Bachelor	General bacteriology (practices) (Colombia and Switzerland), History of science (Biology) (Colombia), and Problem-based learning, APP (Switzerland).

Master	Microbiology in Biogeosciences, BGS (practical), Basic and Advanced molecular biology for BGS students (practices), Molecular ecology techniques (practices), University of Neuchâtel (UNINE) - University of Lausanne (UNIL)
Doctoral	Symposia and seminars on the doctoral programs in Microbiology CUSO – Switzerland, and Doctoral program on Organismal Biology UNINE, Neuchâtel, Switzerland.

Supervising activities

Bachelor	Since 2010 advisor of 5 bachelor students (Stage in General microbiology)
Master	Since 2010 cooperation and supervision of 1 master BGS student

Awards

Dates	2006 Agency: Inter American Economy Bank and Cultural Institute from Mexico & Colombia, sponsored by Museum Banco de la República, Colombia
Award	Discussion article Award - The archetype of Microbial Universe.
Dates	2008 Agency: Colombian Society for Biological Sciences
Award	National Award on Genetics and Biotechnology for my research in bacterial bioplastics

Grants FEMS Grant for Young Scientists for the 5th FEMS Congress of European Microbiologists, Leipzig, Germany 2013

Additional information

Publication record	Since 2011, four publications in peer-reviewed journals, two in submission, four publications in Colombian journals for sciences, and one book in Microbial Eecology (In Spanish, Akademiker -Verlag).
Service related activities	Acted as a reviewer for a manuscript submitted to: Molecules Journal, Societies, and Dyna Journal.
Administration work	Academic duties for Bachelor students in Biology (General Microbiology) at UNINE, 2010-2013 Cooperation in installation duties for the Swiss Microbial Ecology (SME) Meeting 2013, Löwenberg, Murten, Switzerland.

Expertise Microbial ecology, isothermal microcalorimetry, and molecular biology.

Publication list

Peer-reviewed publications (5 years)

2013

Bravo, D., Cailleau, G., Clerc, M., Martin, G., Sharma, A., Verrecchia, E., Junier, P. The oxalate-carbonate pathway and oxalotrophic bacteria: A comparison between tropical forest from India and Cameroon. To be submitted to *Geobiology*

Bravo, D., Braissant, O., Cailleau, G., Verrecchia, E., & P. Junier. Isolation and characterization of oxalotrophic bacteria from tropical soils. To be submitted to *Environmental Microbiology*

Bravo, D., Cailleau, G., Bindschedler, S., Simon, A., Job, D., Verrecchia, E., & P. Junier. A method to isolate oxalotrophic bacteria able to disperse using fungal mycelia. *FEMS Microbiology Letters*. **In Press**

Bravo, D., Martin, G. Cailleau, David, M., Verrecchia, E. & P. Junier. Identification of active oxalotrophic bacteria by BrdU DNA-labeling in a microcosm soil experiments. *FEMS Microbiology Letters*. **In Press**

Revelo, D, Gómez, M., Concha, M., Bravo, D., Fernández, P. Characterization of hydrocarbonoclastic marine bacteria using the 16s rRNA gene. A microcosm study. *DYNA Journal*. **180**: 122-129

2012

Martin, G., Guggiari, M., Bravo, D., Zopfi, J., Cailleau, G., Aragno, M., Job, D., Verrecchia, E., & P. Junier. Fungi, bacteria and soil pH: the oxalate-carbonate pathway as a model for metabolic interaction. *Environmental Microbiology*. **14**:2960-2970

2011

Bravo, D., Braissant, O. Solokhina, M., Clerc, M., Daniels, A.U., Verrecchia, E. & P. Junier. Use of isothermal microcalorimetry assay to characterize microbial oxalotrophic activity. *FEMS Microbiology Ecology*. **78**: 266-274

2009

Bravo, D., Fernandez, P. Sintesis de Polihidroxilacanoatos en bacterias diazotrofas asociadas a leguminosas de la familia Fabaceae en bosques altoandinos de Nariño, Colombia. *Revista de la asociación colombiana de ciencias biológicas* ISSN: 0120-4173. **21**: 11-19

Fernandez, P., Bravo, D. Abundancia de bacterias productoras de Polihidroxialcanoato en suelos de la región andina de Nariño, Colombia. *Revista Centro de Estudios en Salud* ISSN: 0124-7107. **1**: 31-42

Bravo, D., Fernandez, P. Archetipo del universe microscópico. *Revista Centro de Estudios en Salud* ISSN: 0124-7107. **8**: 171-176

Book

Bravo, D., Fernandez, P. Sintesis de PHAs en bacterias diazotrofas en leguminosas de Colombia. Diversidad microbiana funcional. Akademischer Verlag. Saarbrücke, Germany ISBN 978-3-659-06052-6.