RESEARCH LETTER

Isolation of oxalotrophic bacteria able to disperse on fungal mycelium

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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 fungal networks 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 or glass fibers 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.

Introduction

Bacteria and fungi have probably coexisted 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 coexistence 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 produce 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 because fungal synthesis (Lapeyrie, 1988; Tuason & Arocena, 2009) and consumption

(Guggiari *et al.*, 2011) of Caox have 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 bioaccessible 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 toward the comprehension of the role of fungi–bacteria interactions in soil microbial activity and functioning.

Material and methods

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 an 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, six 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.

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 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 (*c.* 20 mL) was prepared from solutions A and B (ratio 100 : 1) without the addition of the carbon source. The upper layer (*c.* 5 mL) was prepared using the same solutions supplemented with Caox. The composition of solution A was (per L): Na_2H-$PO_4.12H_2O$ 9 g; KH_2PO_4 1.5 g; NH_4Cl 1 g; $MgSO_4.7H_2O$ 0.2 g. One millilitre 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 $CaCl_2.H_2O$ 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.

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 with micro invertebrates (strain of successive colonies) was never observed.

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
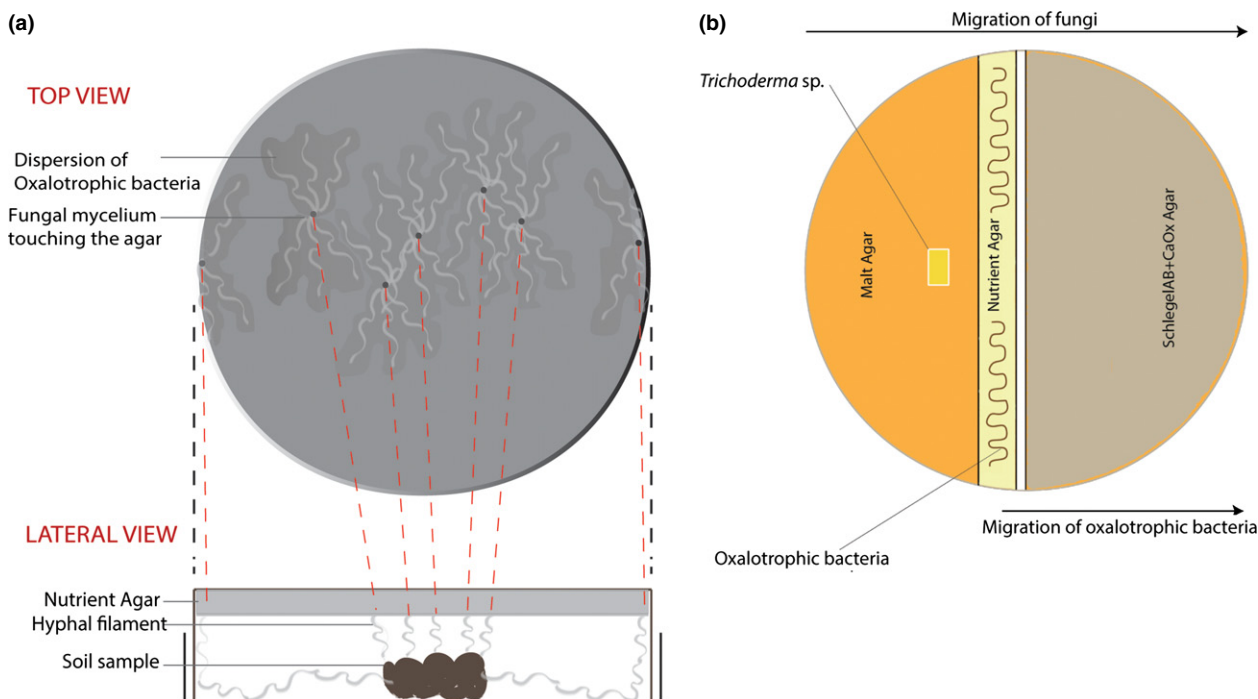
(a)

TOP VIEW

Dispersion of
Oxalotrophic bacteria

Fungal mycelium
touching the agar

LATERAL VIEW

Nutrient Agar
Hyphal filament
Soil sample

(b)

Migration of fungi

Trichoderma sp.

Malt Agar

Nutrient Agar

SchlegelAB+CaOx Agar

Oxalotrophic bacteria

Migration of oxalotrophic bacteria

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 with 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.

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). 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 (1 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.

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 prestep was performed on the biomass at 60 mA during 7 s 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). 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′-GAG-TTTGATCCTGGCTCAG-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 mixtures used for both bacterial and fungal DNA amplification contained (in 50 µL of final volume): 1× Standard Buffer (Biolabs, New England, Ipswich, MA) 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). A total of 2 µL of diluted DNA (*c.* 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 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×.

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 1× 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 16S rRNA gene or the ITS was performed using BLASTN (Altschul *et al.*, 1997) comparing the query sequence with the nonredundant 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′-CTSTAYTTCACSATGCT SAAC-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 2, 2013).

Transport confirmation test

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) 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) 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 zigzag around the NA fragment. Controls consisted in Petri dishes inoculated with oxalotrophic bacteria without the fungal partner, to be sure that bacteria alone do not reach the target medium. 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 out to verify the specificity of the interaction. In this case, the fungus was replaced by glass fibers (Glass wool 8799C Cole-Parmer IL) 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 with the glass fibers using a Leica DMR Trinocular Industrial Microscope, adapted with a digital camera DXM 1200 Nikon (Leica Microsystems AG, Heerbrugg, Switzerland).

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
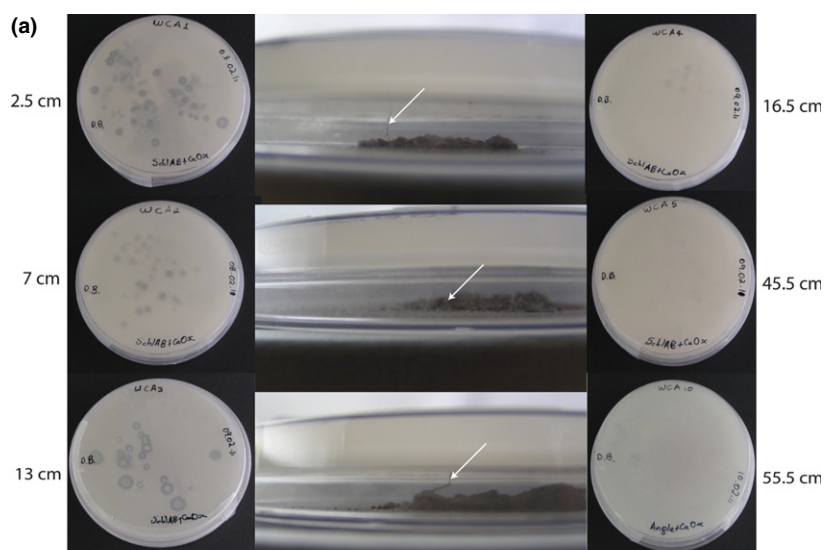

Fig. 2. Quantification of fungus-driven bacterial dispersal associated with the flux of calcium oxalate in soil. (a) Images showing the growth of oxalotrophic bacteria isolated using the inverted Petri dish with Schlegel AB-Caox as target medium. Images from the middle part show the physical contact 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.
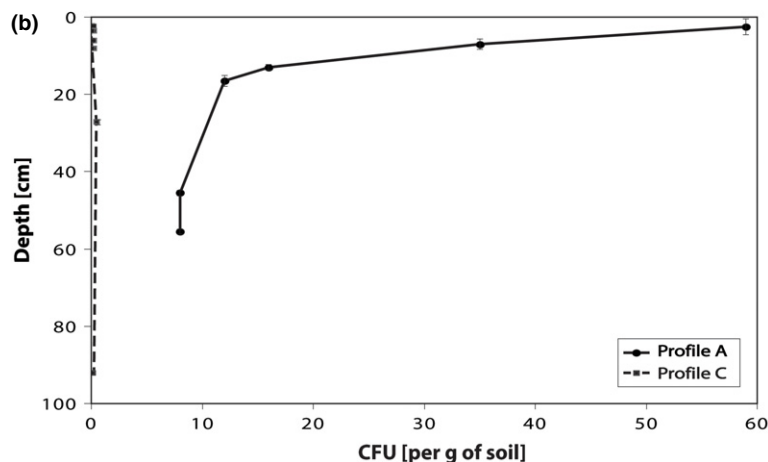
Table 1. Description of eight bacterial strains isolated and characterized using the inverted Petri dish assay and the 'fungal highway' concept for dispersion of oxalotrophic bacteria. Strains were identified by sequencing the 16S rRNA gene. After migration in fungal hyphae, DNA was extracted and the 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 because *Achromobacter* sp. WCA2 and *Pseudomonas* sp. WCA4 did not amplify the *frc* gene

Strain	First hit strain in BLAST	Gram	Flagella*	Class	*frc* gene	After migration 16S rRNA	After migration *frc* gene
WCA1	*Stenotrophomonas* sp.	+	Twitching	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	+	+	+

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

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, 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, for example, 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.

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 d). Consequently, the mechanism of oxalate degradation in *Achromobacter* sp. WCA2 and *Pseudomonas* sp. WCA4 needs to be investigated further.

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 of the eight genera possess flagella and a motility mode (swimming or swarming) associated with 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 vs. 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 mycelial 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).

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)
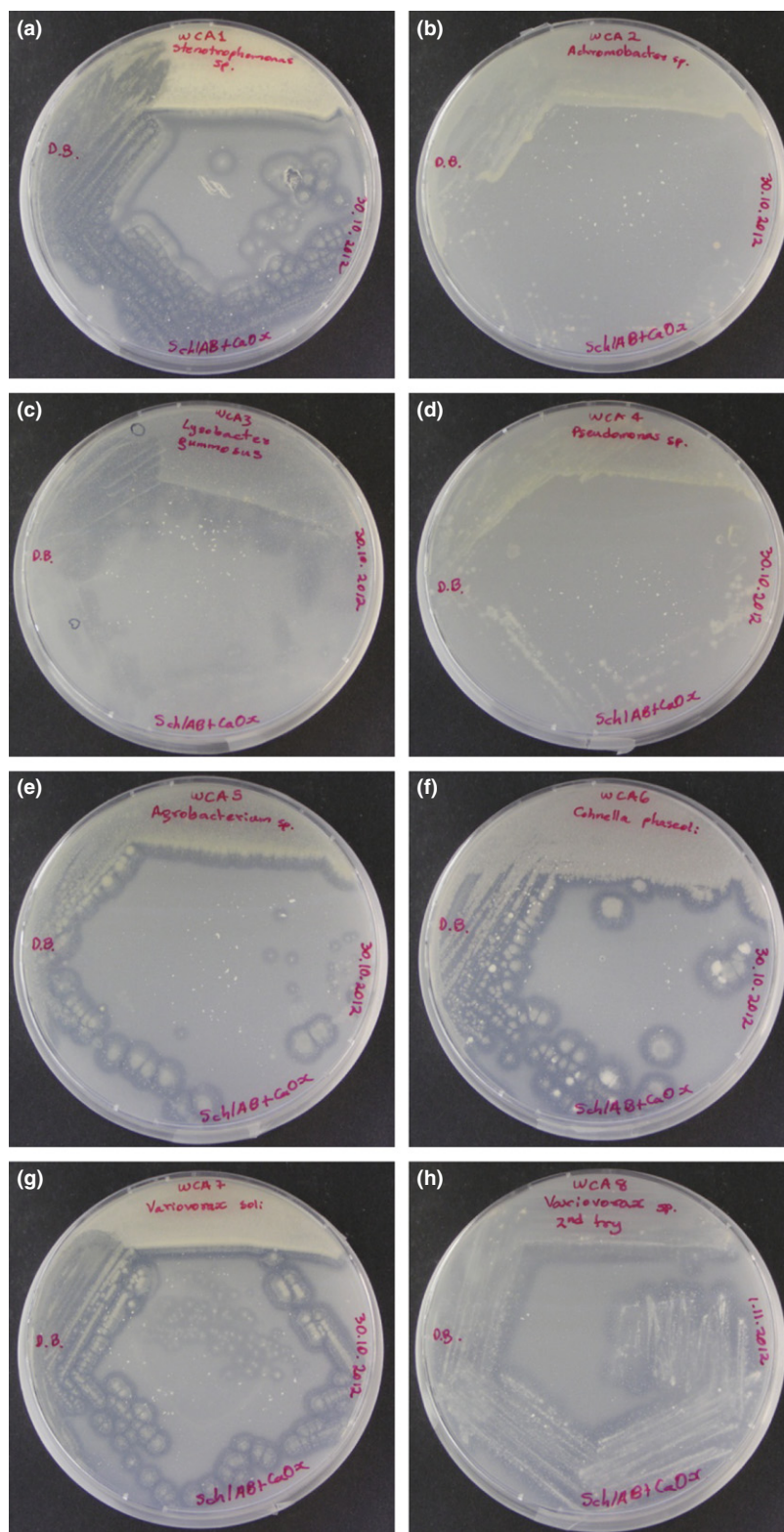
Fig. 3. Degradation halos observed after growth of the bacterial strains isolated by the inverted Petri dish method (see Table 1). The medium corresponds to Schlegel AB medium with calcium oxalate (Caox) as sole carbon source. The degradation halo corresponds to the dissolution of Caox that gives a milky 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.

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
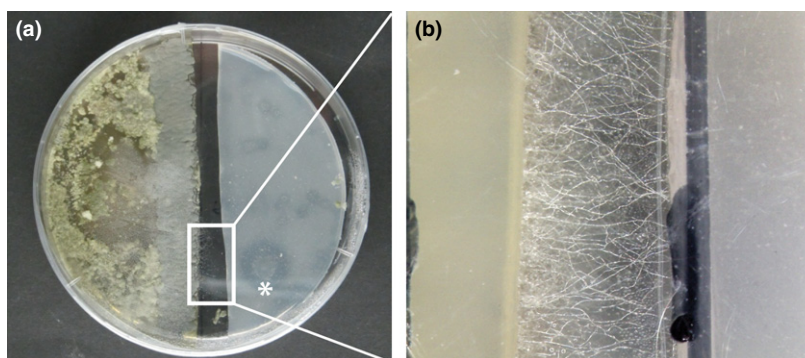
Fig. 4. Test results of bacterial dispersion along fungal hyphae. (a) The interaction with *Trichoderma* sp. allowed the tested oxalotrophic bacteria (here *Stenotrophomonas* sp. WCA1) to access the target Schlegel AB-Caox medium. The dispersion of oxalotrophic bacteria is due to fungal highways formation 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).

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 (Supporting Information, Fig. S1), suggesting that oxalotrophic bacteria clearly take advantage of the physical dispersal network offered in soil by fungal hyphae.

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 to 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 with *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 bacteria–fungi interaction. 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.

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Supporting Information

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

Fig. S1. Test results of bacterial dispersion along fungal hyphae using glass fibers that simulate hyphal filaments.