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REGULAR ARTICLE

Moving carbon between spheres, the potential oxalate-carbonate pathway of *Brosimum alicastrum* Sw.; Moraceae

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Abstract
Aims The Oxalate-Carbonate Pathway (OCP) is a biogeochemical process that transfers atmospheric CO_2 into the geologic reservoir as $CaCO_3$; however, until now all investigations on this process have focused on species with limited food benefits. This study evaluates a potential OCP associated with *Brosimum alicastrum*, a Neotropical species with agroforestry potential (*ca.* 70–200 kg-nuts yr^{-1}), in the calcareous soils of Haiti and Mexico.
Methods / results Enzymatic analysis demonstrated significant concentrations of calcium oxalate (5.97 % D.W.) were associated with *B. alicastrum* tissue in all sample sites. The presence of oxalotrophism was also confirmed with microbiological analyses in both countries. High concentrations of total calcium (>7 g kg^{-1}) and lithogenic carbonate obscured the localised alkalinisation and identification of secondary carbonate associated with the OCP at most sample sites, except Ma Rouge, Haiti. Soils adjacent to subjects in Ma Rouge demonstrated an increase in pH (0.63) and $CaCO_3$ concentration (5.9 %) that, when coupled with root-like secondary carbonate deposits in Mexico, implies that the OCP does also occur in calcareous soils.
Conclusions Therefore this study confirms that the OCP also occurs in calcareous soils, adjacent to *B. alicastrum*, and could play a fundamental and un-accounted role in the global calcium-carbon coupled cycle.

Keywords Oxalate-Carbonate Pathway (OCP) · *Brosimum alicastrum* · Calcium oxalate · Carbon-calcium cycle · Oxalotrophic bacteria

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Study locations Merida, Yucatán Peninsula, Mexico: Oxtapacab (20.77111°N / 89.50417 °W), San Jose Tzal (20.824167 °N / 89.66111 °W), Tzucacab (20.07083 °N / 89.05055 °W) and Haiti: Anse-à-Pitres (18.04306 °N / 71.75833 °W), Anse-Rouge (19.63333 °N / 73.05000 °W).

Highlights
1) Calcium oxalate identified in all analysed *Brosimum alicastrum*.
2) CaOx crystals probably help its younger form augment incident UV-radiation in light-limited environments.
3) Ma Rouge, a Haitian sampling site, demonstrated signs of early onset oxalotrophy.
4) Root-like secondary carbonate deposits were discovered in Mexico.
5) Evidence suggests that *Brosimum alicastrum* is oxalogenic and that oxalogenesis can occur in calcareous environments.

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Introduction

Soils play a major role in the cycling of carbon (C) and understanding the processes that regulate C migration

from one reservoir to the next is of globally recognised significance. The Oxalate-Carbonate Pathway (OCP; Fig. 1) is a biogeochemical cycle that results in the transfer of atmospheric carbon dioxide (CO_2^{Atm}) into the geologic C reservoir within soils, as calcium carbonate ($CaCO_3$). The process probably plays an important role in the regulation of CO_2^{Atm} within the global C cycle (Cailleau et al. 2005; Cailleau et al. 2014) when the source of calcium (Ca) is provided by silicate rocks. OCP has several key components, involving; calcium oxalate (CaOx; $CaC_2O_4 \bullet n\ H_2O$) producing plants, fungi, phytophagous invertebrates, and oxalotrophic bacteria (Cailleau et al. 2004, 2011; Cailleau et al. 2014; Garvie 2006). The first stage commences when CO_2^{Atm} is fixed by RuBisCo during photosynthesis, forming biomass and oxalic acid ($H_2C_2O_4$; Fig 2). Oxalic acid can then be converted into insoluble CaOx crystals ($K_{sp} \approx 10^{-8.5}$; Certini et al. 2000; Monje and Baran 2002; Palak et al. 2012) by plants within specialised cells called crystal idioblasts (Faheed et al. 2013; Franceschi and Nakata 2005; Nakata 2002, 2003). These CaOx crystals are subsequently released during herbivory and decomposition, creating a CaOx pool adjacent to the producing species, in its rhizosphere (Cailleau et al. 2011; Jayasuriya 1955), stomachs of endopedonic species (Bassalik 1913), or within phytoabrasions (Cailleau et al. 2004; Verrecchia et al. 2006). Consequently, this pool of CaOx can then be catabolised by bacteria, labelled oxalotrophic through either the common glycolate- (Bravo et al. 2013; Chandra and Shethna 1977; Tamer and Aragno 1980) or less common serine-pathway (Sahin 2003), precipitating C as $CaCO_3$ and creating a distinct local alkalinisation of acidic soils (Cromack et al. 1977; Fig. 3). Therefore, an active OCP has the ability to biominerally transfer CO_2^{Atm} within the geologic reserve.

Although there have been numerous studies on the OCP, analysis has typically focused on tree species in

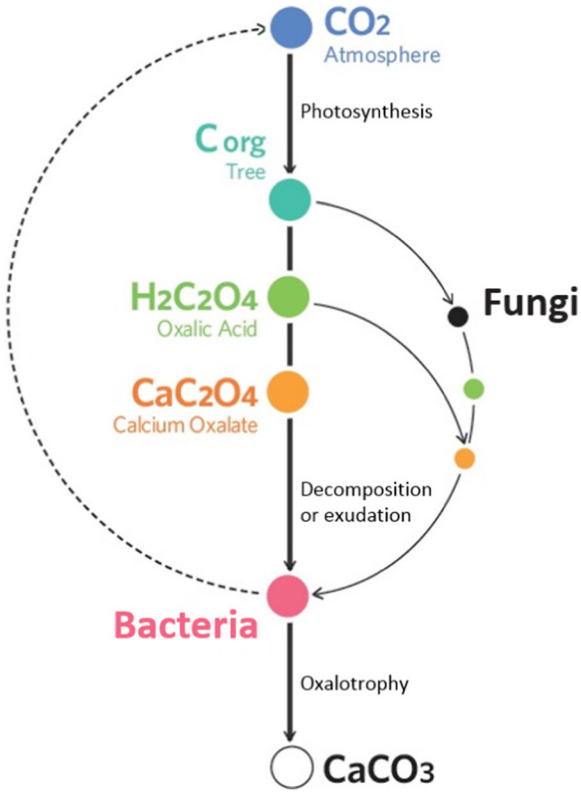

Fig. 1 A simplified model of the Oxalate-Carbonate Pathway (OCP), a process that transfers carbon dioxide from atmosphere to secondary calcium carbonate. As described by Cailleau et al. (2014), the process commences when a calcium oxalate producing species (Tree) organically sequesters carbon during photosynthesis (Corg), converting it into oxalic acid and then calcium oxalate. Once released from organic material during decomposition or as exudes, calcium oxalate is subsequently catabolised by oxalotrophic bacteria (Bact.), converting one mol as carbonate and releasing another as respired carbon dioxide. Fungi also assist in the process by either breaking down oxalic rich matter and depositing calcium oxalate for catabolism by bacteria, or by fungal oxalotrophy

$$\mathbf{1.)\ C_6H_{12}O_6 + O_2 \Longrightarrow 2C_3H_4O_3 + 2\ H_2O}$$

$$\mathbf{2.)\ 2C_3H_4O_3 + 2\ CO_2 \Longrightarrow 2\ C_4H_4O_5}$$

$$\mathbf{3.)\ 2\ C_4H_4O_5 + 2\ H_2O \Longrightarrow 2\ H_2C_2O_4 + 2\ H_4C_2O_2}$$

$$\mathbf{4.)\ 2\ Ca^{2+} + 2\ H_2C_2O_4 + O_2 \Longrightarrow 2\ CaC_2O_4.H_2O}$$

Fig. 2 Oxalic acid production and subsequent precipitation of calcium oxalate from glucose. 1.) Glucose is first oxidated to form pyruvate. 2.) Then pyruvate is carboxylated to produce oxaloacetate. 3.) The subsequent hydrolysis of oxaloacetate forms oxalate and acetate. 4.) Where Ca^{2+} can then react with oxalic acid to form calcium oxalate as either mono- or di-hydrate crystals (Verrecchia 1990; Verrecchia et al. 2006)

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$$1000CaC_2O_4 \,.\, n\,H_2O + 372\,O_2 + 32\,NH_4^+ \Rightarrow 32\,C_4H_8O_2N^+_{Biomass} + 936\,CaCO_3 + 64\,Ca(OH_2)_{aq} \cdots + (1000.(n-2) + 1872)\,H_2O + 936\,CO_2$$

Fig. 3 Oxalotrophic catabolism of calcium oxalate by bacteria (in Verrecchia et al. 2006, from Harder et al. 1974)

acidic soil environments. At current, investigations have confirmed 24 species are associated with active OCPs (Braissant et al. 2002; Cailleau et al. 2004; Cailleau et al. 2014; Ferro 2012; Garvie 2003, 2006; Monje and Baran 2002), typically utilising the emblematic localised alkalinisation of acidic soils as a geochemical proxy for oxalogenesis. The most heavily investigated OCP is associated with *Milicia excelsa Welw.* (Moraceae) in ferralitic soils of Africa (Aragno et al. 2010; Braissant et al. 2004; Braissant et al. 2002; Cailleau et al. 2005; Cailleau et al. 2004; Martin et al. 2012). For which, Cailleau et al. (2011) demonstrated a potential sequestration of *ca.* 1 t C as $CaCO_3$ throughout a model individual's lifetime. Later work identified a further two species within the Moraceae family (Cailleau et al. 2014), associated with an OCP, while earlier work has demonstrated CaOx production in several other species within the family (Wu and Kuo Huang 1997), including the food-producing genus *Brosimum* (Scholz et al. 2007). However, most studies on the OCP have focused on species without agroforestry potential and there has currently been no investigations into a potential OCP associated with the Moraceae genus *Brosimum.*

Brosimum alicastrum Swartz, Moraceae *(B. alicastrum)* is a large Neotropical, ever-green, canopy-emergent tree species utilised in Central America for agroforestry purposes and conservation marketing operations. It is common throughout the dry and wet semi-evergreen forests of the Caribbean, Central America, and Northern-South America (Ortiz et al. 1995; Yates and Ramirez-Sosa 2004). The species has a height range of around 20–40 m, increasing with precipitation, and a common Diameter at Breast Height (DBH) of 1–1.5 m, increasing North–south (Peters 1983, 1989). It is a species shown to be drought resistant (Brewer et al. 2003; Querejeta et al. 2006), growing well in Leptosols of different biomes, while producing nutritious nuts (Peters and Pardo-tejeda 1982). These natural products can be processed to form a range of foods, medicines and excellent fodder for almost all large gregarious mammals, (Gillespie et al. 2004; Ricogray et al. 1991). The species starts seed production after reaching sexual maturity (i.e. 5–7-yr) and, thereafter, an individual can produce around 70–200 kg-seeds yr^{-1} (±30 kg) throughout its 150–200-yr life cycle (Gillespie et al. 2004; Ortiz et al. 1995; Peters 1983, 1989). Furthermore, recent work by Woda and Martinez (2013) has shown that *B. alicastrum*'s seeds have an established, economic harvest return of US $ 650 ha yr^{-1} in Honduras, almost doubling that of maize (US $ 326 ha yr^{-1}); thus, highlighting the potential of *B. alicastrum* as an effective agroforestry crop.

If *B. alicastrum* was found to be in association with an active OCP, it would represent an ideal agroforestry crop with biomineral C fixation capabilities. However, currently the OCP has only been identified in acidic soils, free from inherited carbonate (Cailleau et al. 2014), unlike the predominate habitat of *B. alicastrum* (Peters and Pardo-tejeda 1982). The presence of carbonate in calcareous soils increases the complexity of identifying an OCP (Cailleau et al. 2014), but shouldn't prevent its identification through the analysis of the process' constituents and geochemical proxies. Therefore, the aim of this work is to ascertain if *B. alicastrum* is associated with an active oxalate-carbonate pathway in the calcareous soils of Haiti and Mexico, via the following questions:

1) Does *B. alicastrum* produce CaOx, and if so, is there geochemical evidence of an active OCP adjacent to the species in calcareous soils?
2) Are there oxalotrophic bacterial communities in calcareous soils adjacent to subject *B. alicastrum* in both Haiti and Mexico?
3) What is the C fixation potential of a model *B. alicastrum* agroforestry system in calcareous soils?

Materials & methods

Site settings

Calcareous sample sites were selected with notable environmental and biogeographical similarities in Anse-à-

Pitres (A, B - Banane, C - Bony, D – Ma Rouge) and Anse Rouge (E), Haiti and, the Yucatán Peninsula (F - Tzucacab, G - Oxtapacab, H – San Jose Tzal), Mexico (Fig. 4). The Yucatán sites sit atop a partially emergent carbonate platform of low-lying, Tertiary limestone karst and were typically characterised as either Calcic or Calcaric Leptosols or Cambisols in Oxtapacab and San Joze Tazal, or Luvisols in Tzucacab (Ramos 1975; Shang and Tiessen 2003; WRB 2015) that receive an annual rainfall of approximately 1,100 mm yr^{-1} (Giddings and Soto 2003). Mexico provided mature trees in contrast with Haiti, where only recent plantations were available for sampling. Anse-à-Pitres and Anse Rouge also sit atop Tertiary limestone karst, with thin eroded soils that were predominately classified as Calcaric or Calcic Cambisols, although several sites in Ma Rouge presented a non-calcareous nature (just below classification of Calcaric ≤2 % $CaCO_3$; WRB 2015). Accurate climatic data on the two regions is sparse, but both regions are subject to an annual hurricane season of fluctuating strength (Whigham and Lynch 1998; Whigham et al. 2003), which just preceded sampling for this investigation (2013).

Sampling

A sample of 50 subject *B. alicastrum* of varying size and maturity were selected from both study countries (20 Mexican, 30 Haitian) using stratified-random techniques. Two samples were taken from each subject, an experimental sample from adjacent to the subject and a control sample, exogenous of the subject's zone of lateral edaphic influence (3.5–25 m depending on subject height). To analyse the bulk differences between adjacent and control sites, all soil profiles were taken to their shallow bedrock (10–40 cm) and bulked.

Samples of *B. alicastrum* tissue were taken from each subject for biogeochemical analyses. 3 Foliar and 3 branch samples were taken from the lowest branches of each subject, mid-branch, ensuring uniformity

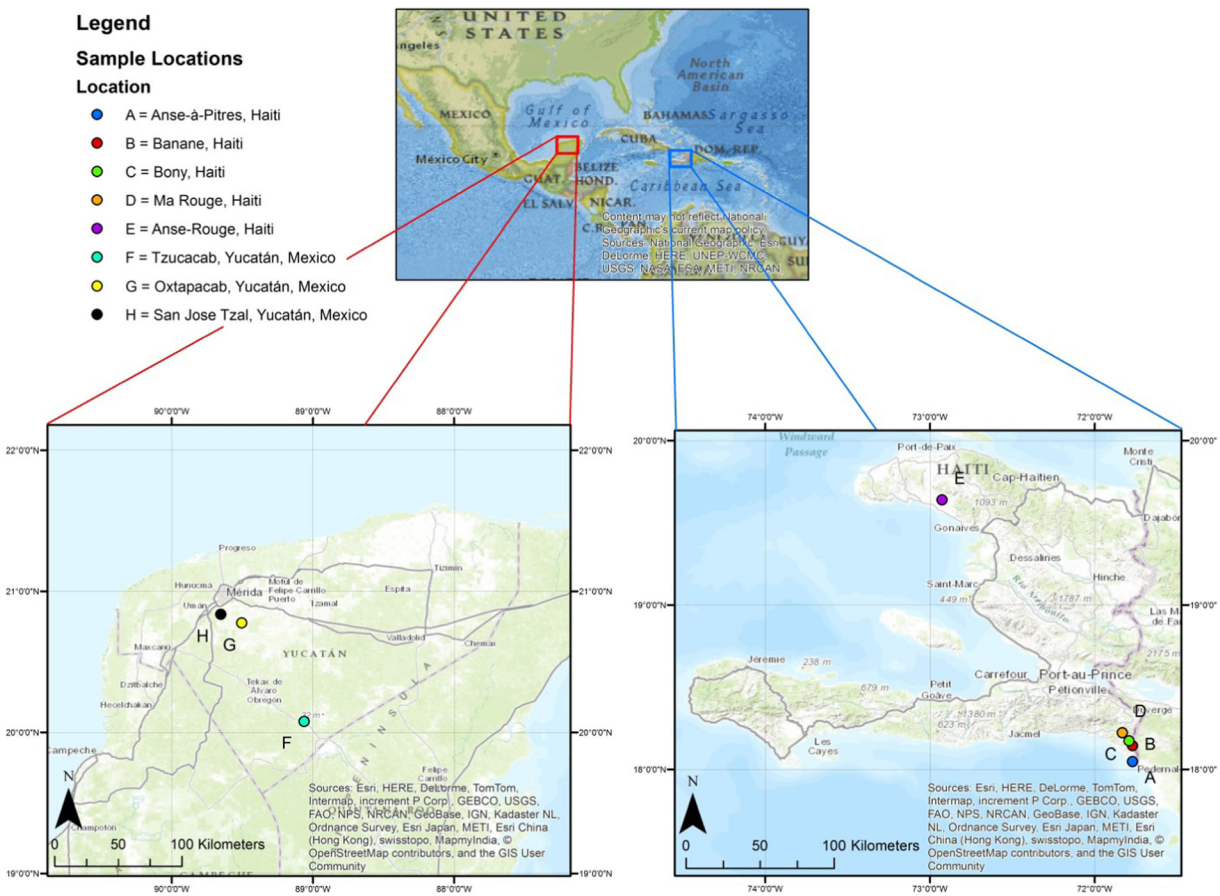

Fig. 4 Sample sites in Haiti and Mexico (Esri 2014)

amongst samples in both countries. Root and bark samples were only taken from Mexican subjects to restrict the damage to the younger trees in Haiti. Subject locations were recorded using GPS systems (Opteka GPS & Garmin GPS 76) and measurements of DBH, soil sample depth, and height, were obtained using 30 m tape and, where necessary, in conjunction with a clinometer (Sokkia No. 8047).

Sample preparation

All soil and plant samples, except those for bacterial analyses, were air-dried in the field to prevent decomposition and decay, and then transported to Bournemouth for laboratory analyses via courier. Bournemouth samples were autoclaved (Astell Swiftlock Securetouch +; 121 °C for 30 mins) on arrival as part of the plant health licence (Food, Environment and Rural Affairs) for importing foreign soils and plant material into the UK. Soil samples were then sieved to fine earth fraction (<2 mm) for chemical analysis, while plant samples were homogenised using a rotor mill and stainless steel-bore mill kit (Retsch MM200). Field-moist samples from each study site, except Anse Rouge, were sent urgently to a laboratory in the Yucatan for bacterial analysis and stored at 4 °C prior to examination. Live samples from Haiti were delayed in Mexican customs for a month, but were also held at 4 °C.

Calcium oxalate analysis

Microscopy

The presence of CaOx in *B. alicastrum* tissue was first identified using optical and Scanning Electron Microscopy (SEM). Samples were prepared for optical microscopy using techniques adapted from Ilarslan et al. (2001). Various tissues from both countries were submerged in Carnoy Fluid (3:1 ethyl alcohol: acetic acid) and left in Petri dishes for 24 h, then re-submerged in ethyl alcohol for 1.5 h. Samples were then coated in 2.5 % sodium hypochlorite and rested for 4 h before mounting with glycerine-gelatine. Slides were observed with an Olympus BX51 compound microscope, using both dark and light field microscopy, and images were captured with an Olympus DP70 Digital Microscope Camera (Olympus Inc.).

SEM and Energy Dispersive X-ray Spectroscopic (SEM/EDS) techniques adapted from Garvie (2003) were used to image and detect the composition of observed crystals. Homogenised plant tissue was applied to alloy stubs using adhesive stickers and AuPd sputter coated (B-7341 Agar Auto Sputter Coater) for 40–60 s. Samples were subsequently analysed in high vacuum using a Jeol JSM-6010 Plus/LV SEM, with an INCA X-sight 8129 EDS system (ETAS Inc.), and InTouchScreen software. All EDS readings represent a percentage of the analysed substance's atomic weight and were recorded in K-band. Furthermore, surficial measurements are considered semi-quantitative as these measurements are applied to 3-dimensional objects.

Enzymatic oxalate analysis

Calcium oxalate concentrations of each *B. alicastrum* sample were quantified using a commercial Enzymatic Oxalate Kit (EOK; Trinity Biotech Plc; Cailleau et al. 2014; Certini et al. 2000). The EOK functions through the oxidation of oxalate by the enzyme (oxalate oxidase) into CO_2 and hydrogen peroxide, which is subsequently oxidized by peroxidase, 3-methyl-2-benzthiazlinone hydrazine (MBTH) and 3-dimethylamino benzoic acid (DMAB) into an indamine dye with a maximum absorbance of 590 nm. Sub-samples of 0.1 g were taken from each plant tissue sample and placed into 30 mL tubes, combined with 5 mL 1 M hydrochloric acid (HCl) extractant and shaken for 16 h at 150 revs min^{-1} (Bibby Stuart Orbital Shaker SO1). The extractants were then centrifuged at 3,000 revs min^{-1} for 5 mins (Heraeus Instruments Megafuge 1.0) and 1 mL supernatant transferred into new 30 mL tubes. This was subsequently combined with 4 mL Ultra-Pure H_2O (Millipore™; 18.2 mΩ at 25 °C) and 0.4 mL 2 M sodium hydroxide (NaOH) for pH correction (pH 5–7) and, thereafter, the manufacturer's instructions were followed. Absorbance was then measured at 590 nm using a Carey 50 UV/vis spectrophotometer (Varian Inc.) after 20 min had elapsed to allow full colour development. Certain soil samples were also measured with the same techniques, adjusting the extraction procedure for the lower concentrations of oxalate. The kits reported the oxalate concentration in mg kg^{-1} which was then adjusted by multiplying the concentrations by the difference in M.W. (1.66) between whewellite (CaOx monohydrate; $CaC_2O_4.H_2O$ M.W.: 142.112 g M^{-1}) and oxalate ($C_2O_4^{-2}$ M.W.: 88.019 g M^{-1}) to give CaOx monohydrate concentrations of each sample.

Total carbon analysis

Total C was ascertained using dry combustion techniques adapted from Wright and Bailey (2001), analysing the tissue of randomly selected *B. alicastrum* subjects from each sampling location. Briefly, triplicates of 1–2 mg of homogenised sample were placed into a tin capsule (Barry and Pinkard 2013; Schutz et al. 2009) and combusted at 1,600 °C in a thermal elemental analyser (Thermo Finnegan FlashEA 1112), standardising peak integration by combusting 2.5-Bis (5–tert-butyl-benzoxazol-2-yl) thiophene (BBOT).

Analysis of edaphic variables associated with the OCP

Loss on ignition

Organic matter content (% OM) of each soil sample was calculated through loss on ignition (Cailleau et al. 2014). 1 g of oven dried (105 °C; Memmert UN 55) soil was furnaced (Carbolite model OAF 11 / 1) at 450 °C for 12 h and the percentage mass loss on ignition calculated.

Soil pH

pH_{H2O} was measured using techniques adapted from Cailleau et al. (2005). 4 g of soil was combined with 10 mL of distilled water ($_d$ H_2O), reposed for 16 h and measured in triplicate with a pH meter (Hach H135 Mini-lab Pro).

ICP-OES

The elemental composition of all soil samples was ascertained using a Vista-Pro CCD Simultaneous Inductively Coupled Plasma-Optical Emission Spectrometer (ICP-OES; Varian inc.) and different extraction methods. Exchangeable concentrations (Ca^{exch} and P^{exch}) were extracted using 1 M ammonium nitrate (NH_4NO_3) extraction technique adapted from MAFF (1986). 0.5 g of soil was combined with 10 mL 1 M NH_4NO_3 in 30 mL polypropylene tubes and shaken for 33 min at 250 revs min^{-1}, the reposed solution was then filtered (Whatman No. 42) and analysed on the ICP-OES. Total concentrations (Ca^{Tot}) were extracted using nitric acid (HNO_3) digestion in a microwave (Anton Parr Multiwave 3000). 0.1 g of soil from each sample was digested with 6 mL 70 % HNO_3 (Fisher Scientific Primar Plus Trace Metal grade) at 200 °C / 20 Bar (800 W), for 30 mins. The microwaved solutions were then filtered (No. 42) and diluted (50 mL) with Ultra-pure H_2O. Quality Control was ensured through the analysis of process blanks and CRM samples (NWRI/INRE TH-2; extraction efficiency $Ca = 100.00$ %, $RSD = 3.98$; $P = 87.18$ %, $RSD = 5.59$).

Soil carbonate

Calcium carbonate concentration was evaluated with a back titration (Cailleau et al. 2014). Briefly, 1 g of soil was combined with 0.25 M Sulphuric acid (H_2SO_4) and then back-titrated with 0.5 M NaOH until a pH 7 was attained. It was not possible to confirm pure presence of $CaCO_3$ using XRD and although a potential error induced by the presence of magnesium carbonate ($MaCO_3$) is small enough to preclude (Cailleau et al. 2005), $CaCO_3$ concentrations are reported as (Ca_{1-x}, Mg_x) CO_3 % D.W..

Identification of oxalotrophy

Oxalotrophic bacterial analysis was completed on each study site, except Anse Rouge, utilising techniques adapted from Braissant et al. (2004). For each study site, 2 g of field moist sample was placed into a 50 mL centrifuge tube and vortexed for 1 min with 20 mL of 1 % sodium hexametaphosphate ($[NaPO_3]_6$), before reposing for a further 20 mins at room temperature. Serial dilutions (10^{-2} a 10^{-4}) were made with 0.9 % sodium chloride (NaCl) solution and then propagated on petri dishes with two layers of media (Aragno and Schlegel 1992). The first layer was a Schegel medium (7 g L^{-1}), while the second layer consisted of Schegel medium with 4 g L^{-1} CaOx monohydrate ($CaC_2O_4.H_2O$), diluted to 10^{-2} or 10^{-4}. Dishes were then incubated at 30 °C for 10–15 days and counted for colonies, every 72 h after the 3rd day of incubation.

Inverse modelling of a potential OCP

The quantity of CO_2^{Atm} captured during OCP bio-induced $CaCO_3$ precipitation associated with an ideal oxalotrophic system was evaluated through the inverse modelling of observed variables and previous literature values (Benjamin et al. 2001; Cairns et al. 1997; Cairns

et al. 2003; Gill and Jackson 2000; Peters 1989), using inverse modelling equations given in the Supplementary information. The model estimates a potential maximum biomineral $CaCO_3$ precipitation and organic C sequestration associated with a *B. alicastrum* OCP by inverse modelling the biochemical characteristics ascertained with the aforementioned methods.

Statistical analysis

Statistical analysis was utilised to evaluate the potential oxalogenesis of *B. alicastrum*. All data was tested for homoscedasticity (Levene's test, $p > 0.05$) and then analysed with partial correlation. Two-way ANOVAs or independent t-tests were applied using IBM SPSS Statistics Version 21, testing the differences between adjacent and control samples, in both countries.

Results

Calcium oxalate analysis

Microscopy

Optical microscopy revealed crystal deposits throughout *B. alicastrum* OM. Prismatic crystals were typically associated with the vascular structure of OM, while druse crystal deposits were associated with the lamina of *B. alicastrum* foliar tissue, from both Haiti and Mexico (Fig. 5). Crystals were present in all forms of sampled *B. alicastrum* tissue (leaf, branch, bark, and root), even in the youngest measured subjects (<0.5 yrs), while in-situ SEM/EDS analyses detected Ca, C, and oxygen in the crystals. Their composition and crystallographic habiti (Verrecchia et al. 1993) confirmed their CaOx monohydrate nature (Fig. 5).

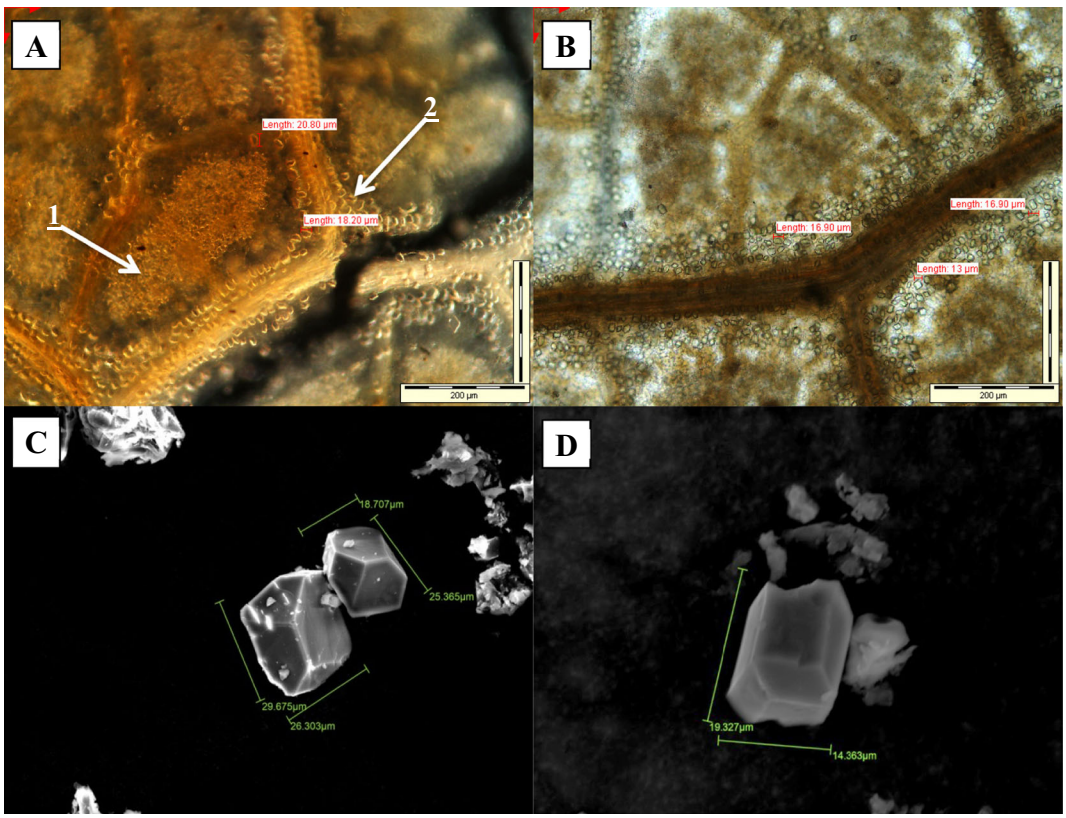

Fig. 5 Calcium oxalate crystals observed and imaged in *Brosimum alicastrum* tissue using optical and scanning electron microscopy. **a** Druse / sand CaOx crystals (1) associated with the lamina, and prismatic CaOx crystals (2) associated with the vascular system of foliar tissue from a Haitian subject. **b** Prismatic and druse CaOx crystals in foliar tissue from a Mexican subject. **c** Prismatic CaOx crystal isolated from the rhytidome of a Mexican subject. **d** Prismatic CaOx crystal isolated from a Mexican subject's root tissue all of which are prismatic habiti common in whewellite

Enzymatic oxalate analysis

Enzymatic oxalate kit analyses quantified the presence of CaOx monohydrate in *B. alicastrum* tissue (Fig. 6). Highest concentrations of CaOx were discovered in Haitian leaf matter, while concentrations decreased with age, between the younger Haitian (mean = 97.26 g kg^{-1}) and mature Mexican subjects (mean = 42.66 g kg^{-1}, $t_{[19]} = 4.385$, $p = 0.001$). High mean concentrations of CaOx were also found in *B. alicastrum* bark (72.79 g kg^{-1}) and root (57.86 g kg^{-1}) material from Mexico, with the lowest concentrations found in branch material of both countries (mean = 38.30 g kg^{-1}). CaOx production did not correlate with Ca^{Exch} ($r^2 = 0.208$, $n = 50$) or P^{Exch} ($r^2 = 0.004$, $n = 50$).

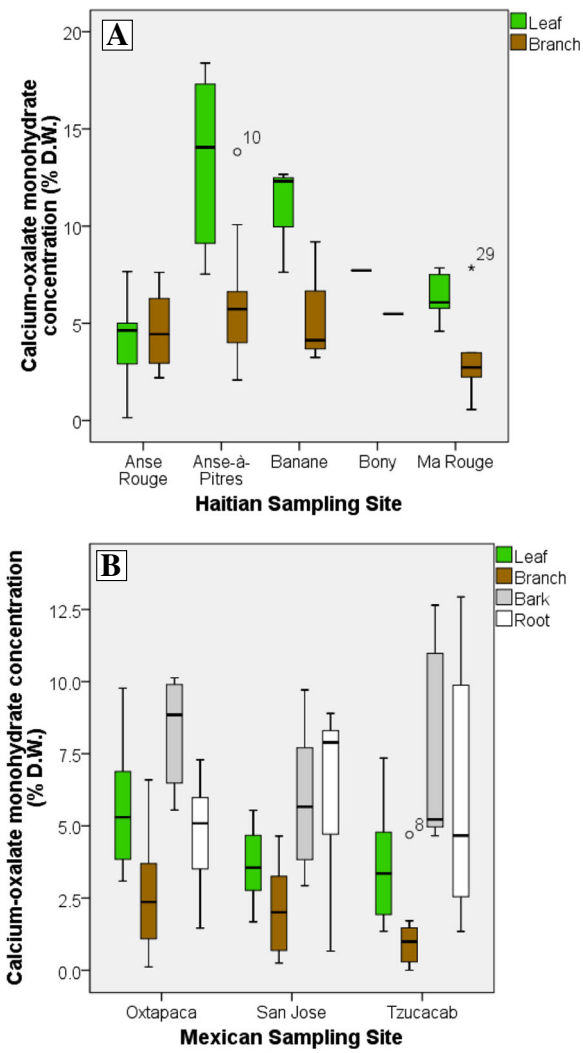

Fig. 6 Box plot graphs displaying oxalate concentrations (% D.W.) of subject Brosimum alicastrum leaf and branch tissue at Haitian sampling sites (**a**), and leaf, bark, root and branch tissue at Mexican sampling sites (**b**)

Analysis of edaphic variables associated with the OCP

The effect of *B. alicastrum* on emblematic edaphic variables associated with the OCP was tested using two-way ANOVAs. The presence of *B. alicastrum* had a negligible effect on all edaphic variables related to the OCP, at all sites combined ($[Ca_{1-x}, Mg_x] CO_3$ $F_{[1,3]} = 0.545$, $p = 0.462$, Ca^{Tot} $F_{[1,\ 3]} = 0.189$, $p = 0.665$ & pH $F_{[1,\ 3]} = 0.07$, $p = 0.787$), except Ma Rouge, Haiti. Ma Rouge displayed the lowest background concentrations of Ca^{Tot} (mean = 6.74 g kg^{-1}) and, although the subjects at Ma Rouge were very young (0.5–2 yrs), the adjacent samples demonstrated clear germinal indications of oxalotrophy (Table 1), namely: (i) a distinct localised alkalinisation, (ii) an increase in concentrations of Ca^{Tot}, (iii; Ca_{1-x}, Mg_x) CO_3 concentration (Fig. 7; Cailleau et al. 2014), and (iv) P^{Exch}, which is unrelated to the OCP, but can be an indicator of CaOx production and release, which subsequently liberates inorganic-bound P (Cannon et al. 1995). There was also an increase in Ca^{Exch} (mean increase 2.73 g kg^{-1}) in the adjacent Ma Rouge sites, but not others ($F_{[1,\ 3]} = 0.002$, $p = 0.962$), indicative of localised Ca cycling by the trees (Jobbágy and Jackson 2001).

Oxalotrophic microbial analysis

Oxalotrophy was detected in cultures from all sampling locations, in both Haiti and Mexico. All samples, except one experimental sample and four control Haitian samples, tested positive for oxalotroph colonies. Haitian study sites displayed a lower frequency of positive colonies than Mexican sites which could be due to the delay in customs; thus, making a direct comparison between the two impossible.

Sampling observations

Multiple mechanisms for the release of *B. alicastrum* produced CaOx were identified in association with subjects in both countries, for instance: phytophagous invertebrate predation (termite) and mycological decomposition (Fig. 8). Secondary carbonate deposits, confirmed through effervescence with 2 M HCl, were found in association with the largest subjects in Mexico. These carbonate deposits were typically concentric, located mid-soil profile, in-between the root network of the subjects, and were

Table 1 Independent samples t-tests comparing the means of edaphic variables related to the oxalate-carbonate pathway, in the adjacent and control profiles at the Ma Rouge sampling site

Variable	Adjacent		Control		t	P	Eta^2
	Mean	SD	Mean	SD			
pH	7.94	0.24	7.31	0.17	5.38	0.00	0.74
Ca^{Tot} (g kg^{-1})	42.79	31.00	6.74	1.91	2.84	0.04	0.45
(Ca_{1-x}, Mg_x) CO_3 (% D.W.)	11.05	4.60	5.15	4.46	2.26	0.48	0.34
P^{Exch} x 10^{-3} (g kg^{-1})	8.09	3.37	1.63	1.40	4.34	0.00	0.65

different in colour, texture and friability from the lithogenic carbonate, crumbling easily upon extraction (Fig. 8).

Carbon capture potential

The calculated values given in Tables 2 and 3 represent an ideal model of oxalotrophy, CaOx production, organic C sequestration, and also decomposition. Whereby, all CaOx and C captured by *B. alicastrum* as either organic C sequestration or $CaCO_3$ precipitation, is stored within the associated C reserve. CaOx concentrations (% D.W.) are calculated from the CaOx concentrations ascertained with the enzymatic oxalate analysis multiplied by the molecular weight of CaOx monohydrate (whewellite; $CaC_2O_4.H_2O$ M.W.: 142.112 g M^{-1}), the most abundant form of CaOx in plants (Aragno et al. 2010).

Discussion

Calcium oxalate and *B. alicastrum*

The present study has demonstrated that CaOx is ubiquitous throughout all forms of analysed *B. alicastrum* tissues, and that production commences at a young age for the species (<0.5 yrs). CaOx concentrations in subject tissue regularly exceeded 5 % D.W. (Libert and Franceschi 1987) and the mean oxalate concentration for all sampled tissues in both Haiti and Mexico was 5.97 % D.W. (59.71 g kg^{-1}, $n = 140$). Furthermore, inverse modelling of the species' biochemical analysis revealed that *B. alicastrum* deposits significant quantities of CaOx into its surrounding edaphic ecosystem on an averaged, annual basis throughout its lifetime. The quantities of oxalate within its tissue are proportionally

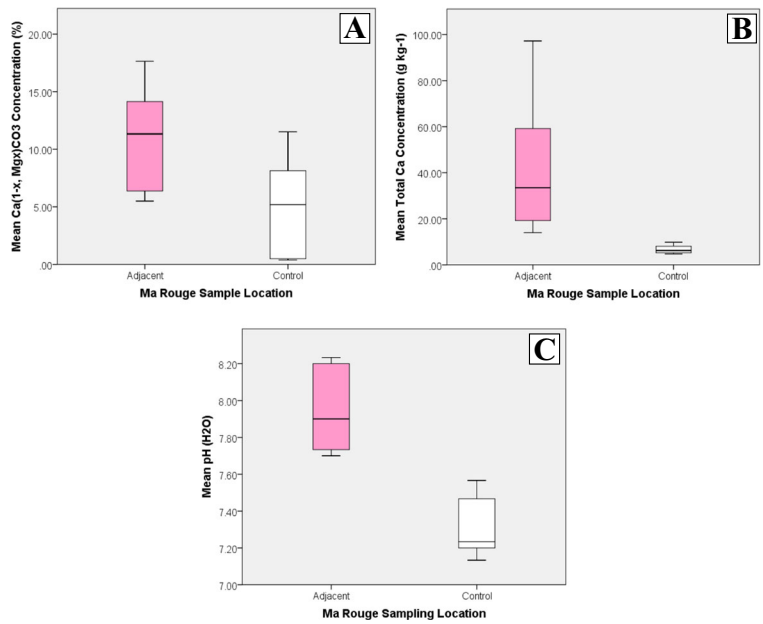

Fig. 7 Box plot graphs displaying soil variables associated with the OCP from adjacent and control (3.5 m distance) samples at Ma Rouge Haiti, which displayed the lowest background concentrations of total Ca, in the following order: (**a**) soil pH values, (**b**) total calcium concentration and (**c**) calcium carbonate concentration, the purity of which was not ascertained

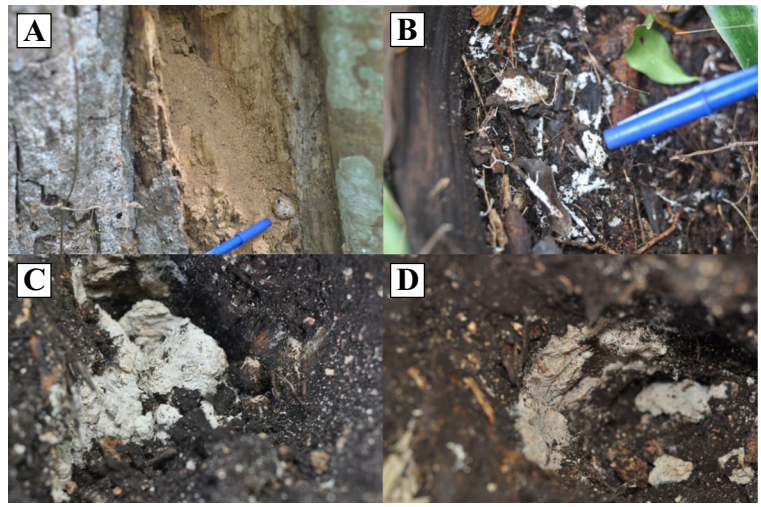

Fig. 8 Photographic observations from sampling. **a** Evidence of phytophagous invertebrate predation. **b** Mycological degradation of CaOx rich tissue in the rhizosphere adjacent to a Mexican subject. **c** Idiosyncratic carbonate mineral deposit, concentric and root-like in structure, located within the root network of a Mexican subject. **d** Concentric carbonate-rich mineral deposit at 1 m distance from a Mexican subject

magnified by the species large biomass; thus, creating a potent source of potential OCP C storage if planted in an acidic soil environment free from a lithogenic carbonate source.

The primary phyto-function of CaOx production in *B. alicastrum* remains unclear. Contrary to previous studies, this investigation found no significant relationship between *B. alicastrum*'s CaOx production and Ca^{Exch} (Austenfeld and Leder 1978; Rasmussen and Smith 1961; Volk et al. 2002) or P^{Exch} (Cannon et al. 1995; Knight et al. 1992) concentrations in most sites, providing weak evidence that CaOx production is utilised for the phyto-regulation of Ca^{Exch} or release of P^{Exch} from inorganic-pools. Equally, the use of *B. alicastrum* tissue for fodder and the non-raphide morphology or size of crystals (Sakai et al. 1984; Salinas et al. 2001) indicate that the species does not use CaOx production as an herbivory deterrent. However, the concentrated production of druse crystals in the lamina of *B. alicastrum* foliar tissue could distribute UV light to chloroplasts, increasing incident UV absorbency in understory environments, as originally hypothesised by Franceschi (2001) and later demonstrated experimentally by Kuo-Huang et al. (2007) in *Peperomia glabella*. This hypothesis explains the observed decrease in subject foliar CaOx concentration with age, while also explaining *B. alicastrum's* high survival rates under dense canopy (>80 %; Laborde and Corrales-Ferrayola 2012). Therefore, a role for *B. alicastrum* CaOx druse crystal production in the maximisation of incident UV light is hypothesised.

Table 2 Mean calcium oxalate and carbon contents of *B. alicastrum* tissue from both countries used in the inverse modelling of the carbon capture ability of an ideal individual or hectare population

Tissue type	Mean calcium oxalate content (% D.W.)	Mean total carbon (% D.W.)
Leaf	7.54	35.68
Branch	3.91	41.63
Bark	7.28	45.70
Root	5.79	41.34
Mean tissue	5.97	39.98

During this investigation, the root network of *B. alicastrum* was of particular interest. *B. alicsatrum* has a root network that is mainly concentrated in the upper soil and bedrock layers (Querejeta et al. 2006). The EOK analyses indicated that *B. alicastrum* root tissue contains a significant concentration of CaOx, which, when coupled with the Cairns et al. (1997) root / shoot ratio (0.26) and Gill and Jackson (2000) root turnover rate (0.1 yr^{-1}), predict that *B. alicastrum* deposits significant quantities of CaOx directly into its rhizosphere through the continuous decomposition and regeneration of root OM. Furthermore, investigations have demonstrated that *B. alicastrum* roots have strong associations with mycorrhizal fungi (Allen et al. 2003; Allen et al. 2005) that, Bravo et al. (2013) demonstrated act as highways for the dispersal of oxalotrophic bacteria to oxalate, creating an ideal mutualistic habitat for oxalotrophy.

Table 3 Estimates for the carbon capture ability of an ideal *B. alicastrum* individual and hectare plantation of 400 individuals

Predictions	Total calcium oxalate output (kg)	Potential biomineral precipitation of captured CO_2^{ATM} as $CaCO_3$ (kg)	Potential organic carbon sequestration of CO_2^{Atm} as biomass (kg)	Potential total CO_2 capture (kg)	Annual CO_2 capture (kg yr^{-1} MLE^{-1})
Individual	1590	479	39,633	40,112	267
1 ha plantation (400 individuals)	636,000	191,600	15,853,200	16,048,800	106,800

OCP and carbonate soils

All the constituents of an active OCP in ferralitic soils have now been identified by this investigation in calcareous ecosystems adjacent to *B. alicastrum* in both Haiti and Mexico. These constituents include: an oxalate producing species (*B. alicastrum*), phytophagous invertebrate predation and mycological decomposition of CaOx rich tissue, significant oxalotrophic bacterial communities, and secondary $CaCO_3$. Furthermore a calcareous sample site in Haiti, Ma Rouge, has demonstrated clear, emblematic, early indications of oxalotrophy adjacent to subjects (Table 2) even though the subjects are still very young (0.5–2 yrs). This was contrary to our hypothesis that the trees would be too young to have affected their local edaphic ecosystem; but, at the time of sampling, the Ma Rouge trees had already grown to 1.4–1.8 m in height and were producing significant quantities of CaOx, which was also detectable in the soils adjacent to them (5–25 g kg^{-1}). As demonstrated by Bravo et al. (2011), this soil CaOx pool can be catabolised quickly upon entry into the edaphic ecosystem when in the presence of oxalotrophs. Which, when coupled with positive identification of oxalotrophy in soils found adjacent to *B. alicastrum* in Ma Rouge, strongly suggests that, like suggested by Verrecchia et al. (1993), an OCP can occur in calcareous environments and secondary carbonate deposits found in association with the root networks in Mexico are generated through an active OCP.

Although there was evidence of oxalotrophy in Ma Rouge, typical edaphic variables associated with the OCP were suppressed in most sites. This could be because of the higher concentrations of Ca (Ca^{Exch} & Ca^{Tot}) masking the typical indicators of an OCP. Ma Rouge displayed the lowest concentrations of Ca (Ca^{Exch} & Ca^{Tot}) or $CaCO_3$ (2 sites below the calcaric threshold) of any site samples. The site was also the only site to present evidence of Ca^{Exch} cycling by the plants (Jobbágy and Jackson 2001). However, the passive cycling of Ca could not explain the observed increase in $CaCO_3$ content of adjacent samples. On the contrary, there was a larger increase in adjacent concentrations of Ca^{Tot}, relative to Ca^{Exch}, which, as a plant nutrient would be actively cycled by plants. This increase is most likely linked to the $CaCO_3$ increase adjacent to the species, as will be the localised alkalinisation. A significant saturation of exchange complex by Ca (>4.47 g kg^{-1}) of a deprotonated alkaline soil would typically suppress the localised alkalinisation associated with an OCP in ferralitic environments; but, the observed increase in $CaCO_3$ would further increase pH as seen in Ma Rouge. Although the presence of lithogenic $CaCO_3$ makes it difficult to discern secondary $CaCO_3$ deposits and thereby, identify an active OCP (Cailleau et al. 2014), the root-like position, colour, shape, texture and friability of secondary $CaCO_3$ deposits in Mexico were all suggestive of an OCP associated with *B. alicastrum*. Therefore, Ca and C cycling of the OCP in calcareous environments needs to be studied in more detail to identify alternate indicators of the process in alkaline soils.

In calcareous environments, plants under stress from high Ca^{Exch} concentrations, typically increase CaOx production as a Ca detoxification mechanism (Austenfeld and Leder 1978; Molano-Flores 2001; Rasmussen and Smith 1961; Volk et al. 2002; Webb 1999). This increased production of CaOx would theoretically lead to a larger pool available for oxalotrophy relative to a ferralitic environment, subsequently increasing the C cycling of the process. However, an identifiable C sequestration of a calcareous OCP must be ruled out because CO_2 is concomitantly released into the soil matrix when Ca^{2+} is liberated during the

$$2H^+ + CaCO_3 \Leftrightarrow Ca^{2+} + CO_2 + H_2O$$

Fig. 9 The acidic dissolution of limestone frees Ca in the soil solution for the OCP, but also degases carbon dioxide. This supposes the presence of strong acids. However, for the details of the general balance of the OCP involving calcium carbonate, calcium oxalate, and CO_2, please see Verrecchia et al. (2006)

dissolution of $CaCO_3$ in calcareous environments (Fig. 9). This means that a calcareous OCP system cannot truly be considered a C sink, but instead a C capturer, as the allochthonous, non-carbonate origin of the Ca^{2+} precipitated as $CaCO_3$ cannot be confirmed in this complex system. Instead, this work has confirmed that *B. alicastrum* is an oxalogenic species which is known to have significant agroforestry potential (Woda and Martinez 2013) and if planted in a location free from lithogenic $CaCO_3$, would act as an efficient agroforestry and C capture tool.

Conclusion

Calcium oxalate production takes place throughout *B. alicastrum* tissue and the compound likely plays an important role in the species' adolescent form, maximising photosynthesis, through the augmentation of incident UV radiation in the lamina, in light-limited environments. This research has also identified oxalotrophic bacterial communities in soils from Haiti and Mexico, providing further evidence for previous suggestions that oxalotrophism is globally diverse. Furthermore, this study has provided experimental evidence for the hypothesis of Verrecchia et al. (1993) that, the OCP can occur in calcareous environments. Thus, when planted in soils free from lithogenic carbonate, *B. alicastrum* would represent a valuable C sequestration and agroforestry crop which would have the ability to biomineralally sequester C via an active OCP, while providing food for Neotropical communities in countries such as Haiti, Mexico or Belize. Further investigation is now required to:

1. Analyse *B. alicastrum* in acidic soil environments,
2. Assess the isotopic signatures of discovered carbonate deposits,
3. Assess the origin of Ca sources in calcareous OCP systems,
4. Identify more oxalogenic species with significant agroforestry potential, to facilitate integration of this biogeochemical C management solution into current agroforestry systems.

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