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New insights into the functions of carbon–calcium inclusions in plants

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Summary

Carbon–calcium inclusions (CCaI) either as calcium oxalate crystals (CaOx) or amorphous calcium carbonate cystoliths are spread among most photosynthetic organisms. They represent dynamic structures with a significant construction cost and their appearance during evolution indicates an ancient origin. Both types of inclusions share some similar functional characteristics providing adaptive advantages such as the regulation of Ca levels, and the release of CO_2 and water molecules upon decomposition. The latter seems to be essential under drought conditions and explains the intense occurrence of these structures in plants thriving in dry climates. It seems, however, that for plants CaOx may represent a more prevalent storage system compared with $CaCO_3$ due to the multifunctionality of oxalate. This compound participates in a number of important soil biogeochemical processes, creates endosymbiosis with beneficial bacteria and provides tolerance against a combination of abiotic (nutrient deprivation, metal toxicity) and biotic (pathogens, herbivores) stress factors. We suggest a re-evaluation of the roles of these fascinating plant structures under a new and holistic approach that could enhance our understanding of carbon sequestration at the whole plant level and provide future perspectives.

Introduction

The occurrence of carbon–calcium inclusions (CCaI) for both plant and animal kingdoms has been reported by pioneer anatomists as far back as the 70th century. Calcium oxalate (CaOx) crystals (Fig. 1b–d) and calcium carbonate ($CaCO_3$ lime) cystoliths (Fig. 1a) are common cellular solid deposits in plants and represent biomineralisation (Bauer *et al.*, 2011; He *et al.*, 2013). The evolution of biomineralisation has been linked to the formation of primitive organisms and the diversity of materials, mechanisms and strategies used by an impressive diversity of biomineralising life forms. In this regard, Skinner & Jahren (2003) raised the question 'why biomineralise?' and suggested the answer lies on the two main adaptive advantages afforded by this process: *Physical*, by construction of skeletal components and *Chemical*, by providing a dynamic storage system of essential components. Obviously, the first advantage concerns mainly animal biominerals such as bones, whereas the second one concerns predominately plant biomineralisation, in which case the storage system has to be dynamic to have the ability to offer back to the organism the stored materials. Of course this generalisation is not the rule in every case; for example bones serve as a reservoir for and a source of calcium for critical metabolic needs in mammals (Ross *et al.*, 2011). Conversely, some biominerals, for example, CaOx crystals, do not always act as a source of calcium (Paiva, 2019) or offer physical protection against herbivory. Paradoxically, although $CaCO_3$ is the dominant biomineral in many large organismal groups, its presence in the Plant Kingdom is rather limited (Bauer *et al.*, 2011). By contrast, CaOx is the most prevalent and widespread biomineral in plants (Franceschi & Horner, 1980; Horner & Wagner, 1995), with reduced occurrence in other organisms. It seems therefore that CaOx may represent a more prevalent storage system for plants compared with $CaCO_3$. Taking into account that the inorganic part of both CCaI types is calcium, an inevitable question arises: why CaOx prevailed in the Plant Kingdom?

In this review we provide a general overview of the evolution of CCaIs in photosynthetic organisms and then we compare the two types of plant CCaIs in an attempt to answer this intriguing question. We summarise recent findings regarding CCaIs and provide some new insights by redirecting attention to the functionality of their carbonaceous ion (oxalate or carbonate). Finally, we suggest a re-evaluation of the roles of these fascinating

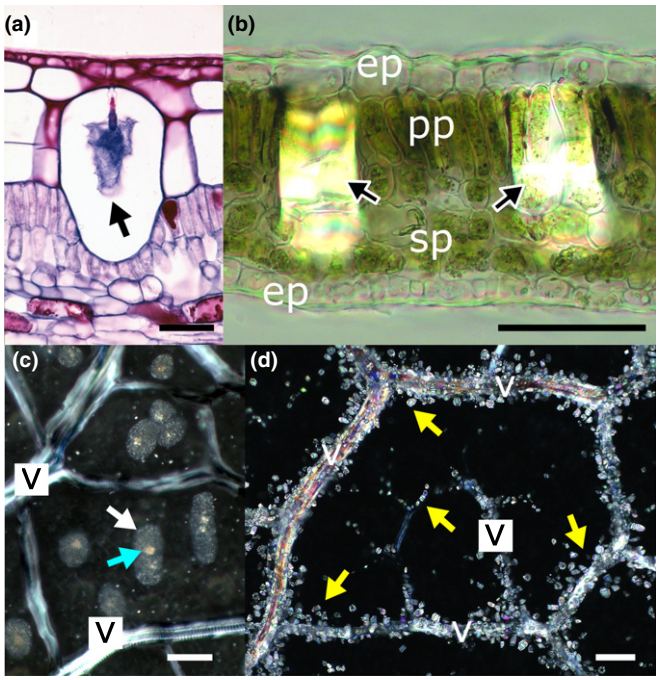

Fig. 1 (a) *Ficus elastica* (Moraceae) leaf lithocyst. $CaCO_3$ was dissolved during fixation of the sample and the cellulosic stalk of the cystolith is obvious (black arrow). Bar, 50 μm. (b) *Punica granatum* (Punicaceae) leaf vibratome cross-section showing two large mesophyll prismatic crystals (arrows). ep, epidermis; pp, palisade parenchyma; sp, spongy parenchyma. Bar, 50 μm. (c) *Uncaria canescens* (Rubiaceae) leaf mesophyll idioblasts (white arrow) each containing crystal sand and a druse (light blue arrow), the scale bar corresponds to 50μm. (d) *Fagus* sp. (Fagaceae) leaf showing prisms (yellow arrows) associated with veins (v) and in mesophyll. Bar, 100 μm. (b) Observed between partially and (c, d) completely crossed polarisers. Bar, 50 μm.

plant structures under a new and holistic approach that could enhance our understanding of carbon sequestration at the whole plant level and provide future perspectives.

Occurrence of CCaIs in photosynthetic organisms is an ancient trait

When taking into consideration the evolution of CCaIs, beginning with the first photosynthetic organisms and leading to the angiosperms, it is important to examine the origin and environmental availability of the two parts of CCaIs. Calcium, the third most abundant metal in nature was adopted as a regulator early in evolution (Carafoli & Krebs, 2016). A probable reason for the choice was its ability to create insoluble, metabolically inactive precipitants and to reversibly bind to specifically developed molecules. It is therefore not surprising that calcium-bearing minerals comprise *c.* 50% of biominerals (Weiner & Dove, 2003).

Concerning the carbonaceous parts of CCaIs, carbonate can be of both environmental and biological origins, but oxalate is exclusively of biological origin. Cellular oxalate formation can occur via several metabolic pathways as reviewed by Franceschi & Horner (1980) and more recently by Igamberdiev & Eprintsev (2016) and Cai *et al.* (2018). The three most significant pathways reported are:

(**1**) from oxidation of glyoxylate. Glyoxylate can be formed during photosynthesis/photorespiration as a product of the glycolate oxidase reaction (glycolate is oxidised to oxalate) or of the isocitrate lyase reaction (isocitrate is converted to glyoxylate and succinate).
(**2**) from oxidative C2/C3 cleavage of L-ascorbic acid via several steps. An intermediate metabolite of this path is dehydroascorbic acid (Zhang *et al.*, 2019).
(**3**) from oxidative cleavage of oxaloacetate (derived either from the PEP carboxylase reaction or from citrate), the reaction catalysed by oxaloacetate acetylhydrolase (Cai *et al.*, 2018).

All three pathways of oxalate formation have been variously reported for both photosynthetic (Cai *et al.*, 2018) and nonphotosynthetic organs of plants (i.e. roots; Horner *et al.*, 2000; to be described later in the section on Occurrence of CCaIs must be considered on a holistic basis) and, at least in spinach, all putative genes involved were functional (Cai *et al.*, 2018).

Considering that in very early life evolution, carbonates were an abundant and readily accessible environmental resource, it is plausible that $CaCO_3$ deposition preceded CaOx deposition. Hence, it is not surprising that $CaCO_3$ deposition occurred in unicellular organisms (bacteria, cyanobacteria and coccolithophores), whereas there is no evidence for CaOx deposition (Pueschel, 2019). Indeed, the extracellular passive precipitation of $CaCO_3$ (calcification) is widespread in these primitive unicellular organisms, offering adaptive advantages (Castanier *et al.*, 2000). Intracellular deposition, contrary to extracellular deposition, is an active, energy-consuming mechanism offering better control of all related processes (Cam *et al.*, 2018). Recently it was found that several species of photosynthetic bacteria (cyanobacteria) form intracellular $CaCO_3$ granules (Benzerara *et al.*, 2014) with unknown roles. The deposition of $CaCO_3$ in the form of coccoliths is also found in coccolithophores, a widely distributed group of marine phytoplankton (Monteiro *et al.*, 2016). The spread of $CaCO_3$ deposition among spermatophytes is rather limited, however mainly in four members of the order Urticales for example Cannabaceae, Moraceae, Ulmaceae and Urticaceae, it is found as an encrustation on cell walls or in an unusual deposit called cystolith (Fig. 1a). Cystoliths are typically located in enlarged surface cells of leaves called lithocysts, in which $CaCO_3$ is deposited over a cellulosic stalk hanging from the cell wall (Bauer *et al.*, 2011, see Fig. 1a).

While studies on fossils have revealed that $CaCO_3$ deposition by photosynthetic microorganisms dates back to 3.3 Bya (Tice & Lowe, 2004), data for CaOx deposition are limited to only extant taxa, as CaOx is not preserved in extinct taxa the initial occurrence of CaOx crystals is unknown. From an evolutionary standpoint, CaOx crystals seem to have arisen in both marine and fresh-water algae, first, after the anion oxalate became metabolically available and the gene(s) controlling oxalate formation may have been passed on to land plants. Probably the first function of oxalate in land plants concerned weathering. According to Igamberdiev & Lea (2006), oxalate formation is linked to the appearance of land plants and is also metabolically linked to a high O_2/CO_2 planet atmosphere. Probably the initial production of oxalate started as a side product of the glycolate oxidase reaction due to the appearance of photorespiration (Igamberdiev & Lea, 2002). The photosynthetic activity of land plants (and green macroalgae,

Pueschel, 2019) is directly connected to the weathering process caused by the excretion of citrate, malate and oxalate from roots, because of the requirement of phosphate. The early land plants sporadically formed CaOx, however, the proliferation of the ferns, gymnosperms and angiosperms provided genetic avenues for CaOx formation and its involvement in a variety of functions. CaOx appears in extant red, green and siphonous algae, fungi, lichens, one bryophyte and lycophytes, increased in ferns and is common in gymnosperms and angiosperms. The variety of forms of CaOx displayed throughout photosynthetic organisms seems to have arisen independently (Anthoons, 2017; Supporting Information Table S1) and in a number of cases there is evidence of phylogenetic relationships (Horner *et al.*, 2015). There is no comprehensive study, to date, that deals with the evolution and types of crystals across the angiosperms. Moreover, oxalate accumulation seems to be independent of photosynthetic pathway (Zindler-Frank, 1976), although recent evidence shows that in some cases it depends on the particular genotype (Miyagi *et al.*, 2019).

Occurrence of CaOx crystals at the interspecific level is related to dry climates

According to the available data involving different sites, a significant percentage (15–53%) of the species in rain forests have leaves with CaOx crystals. This percentage is increased in montane forests (76–86%) and is even higher in xerophytes and poikilohydric organisms such as lichens (Table S1). Some succulents may accumulate enormous quantities of CaOx, for example the total biomass of a large *Carnegiea gigantea* cactus in southwestern Arizona may contain *c.* 100 kg CaOx (Garvie, 2006). Moreover, among different desert species (psammophytes) the drought-resistant plants bear more CaOx crystals compared with grassland plants and herbs (Ci *et al.*, 2010; Table S1). Thus, the occurrence of CaOx crystals seems to constitute a drought tolerant trait and most importantly a key trait of desert plants (Ci *et al.*, 2010). The correlation analysis between the per cent of species bearing crystals and the mean annual precipitation of each sampling site, based on the data of the references providing climatic data in Table S1, revealed a strong statistically significant relationship ($r = -0.79$, $P < 0.01$), which further supports this trend at the interspecific level (Fig. 2). However, in order to fully confirm the association between the occurrence of CaOx crystals and the dry climates, future studies should also consider other missing factors that affect the production of crystals, such as soil calcium availability and transpiratory rates, which interfere with calcium translocation. At the intrageneric level, Brown *et al.* (2013) observed that among different species of *Acacia*, crystals are more abundant in acacias growing in low rainfall areas, compared with those growing in higher rainfall areas. There are also links between the accumulation of CaOx crystals in tree rings and seasonal drought fluctuations (Gourlay & Grime, 1994).

Some similar characteristics of CCaIs

(**1**) Both CCaIs are calcium and carbon pools in a solid form that are metabolically and osmotically inactive without obstructing cell functions.

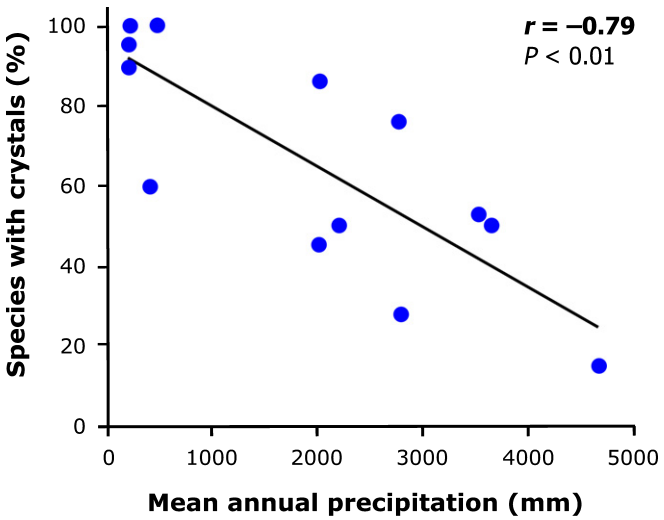

Fig. 2 Correlation between the mean annual precipitation and the per cent of species bearing calcium oxalate (CaOx) crystals from different sites of global distribution. The correlation was based on the species and the climatic data from the references in Supporting Information Table S1. For Cactaceae and the succulents a 200 mm mean annual precipitation was presumed.

(**2**) The decomposition of both CCaIs can produce Ca ions, CO_2 and H_2O. For CaOx, crystal dissolution is a prerequisite for further decomposition.

CaOx dissolution

$$CaOx \rightarrow Ca^{2+} + \text{oxalate ions} + xH_2O^* \quad \text{Eqn 1}$$

CaOx decomposition

$$\text{Oxalate} + O_2 + 2H^+ \rightarrow 2CO_2 + H_2O_2 \text{ (oxalate oxidase)} \quad \text{Eqn 2}$$

$$2H_2O_2 \rightarrow 2H_2O + O_2 \text{ (catalase)} \quad \text{Eqn 3}$$

Sum of Eqns 1, 2 and 3.

$$CaOx \rightarrow Ca^{2+} + 2CO_2 + O_2 + xH_2O^{**} \quad \text{Eqn 4}^{***}$$

Cystolith dissolution/decomposition

$$CaCO_3 + 2H^+ \rightarrow Ca^{2+} + CO_2 + H_2O \quad \text{Eqn 5}$$

*In Eqn 1, water molecules are either zeolitic (mobile) or embedded in the crystal. The number of these molecules depends on the structure of the CCaIs.

**In Eqn 4 water molecules may be zeolitic, embedded in the crystal or produced by Eqn 3.

***An alternative pathway for the degradation of oxalate involves four enzymes, oxalyl-CoA synthetase, oxalyl-CoA decarboxylase, formyl-CoA hydrolase and formate dehydrogenase (Foster *et al.*, 2016; Yang *et al.*, 2018).

The mechanisms of crystal or cystolith dissolution remain unknown, but a drop in idioblast pH combined with the presence of Ca-binding proteins (see Webb, 1999; Leszczuk *et al.*, 2019) or the presence of substances that will form soluble complexes with either calcium or oxalate ions, such as citric acid and magnesium are possible mechanisms. Note that decomposition reactions (Eqns 2

and 5) of both CCaIs require the presence of protons and, for Eqn 2, oxygen.

(**3**) Both CCaIs are dynamic storage systems that can, under certain circumstances, reduce or increase their volumes. Seasonal (Tooulakou *et al.*, 2016 and the literature therein) and diurnal (Tooulakou *et al.*, 2016; Giannopoulos *et al.*, 2019) fluctuations of CaOx crystals and cystolith dimensions have been reported. During diurnal fluctuations the mean volume of the CCaIs decreased until midday, whereas a complete recovery was attained during the late hours of the photoperiod and during the night. Changes in CaOx crystal dimensions were observed in response to changes in Ca concentration of the growth medium (Franceschi, 1989).

(**4**) Irrespective of the type of inclusion, their formation and maximum density occur at very early leaf developmental stages. Inclusion properties are changing in a coordinated way with leaf area, so that each crystal cell or lithocyst 'services' a finite number/area of adjacent cells (Giannopoulos *et al.*, 2018). Moreover, in many leaves, lithocysts or crystal cells are strategically placed either among photosynthesising parenchyma or in bundle sheaths and the amount of sequestered carbon in these structures is considerable (Figs 1, 3).

(**5**) Measurements of $\delta^{13}C$ showed that at least in some plant species (Rivera & Smith, 1979; Tooulakou *et al.*, 2016) and lichens (Beazley *et al.*, 2002), CaOx crystals are less depleted in ^{13}C than the bulk organic C in the biomass. This means that carbon atoms in the CaOx crystals are derived from sources other than the photosynthetic CO_2 assimilation through the atmosphere. Recent findings confirmed that this is also the case for the cystoliths (Giannopoulos *et al.*, 2019).

Similar functions of the CCaIs related to similar characteristics

Calcium homeostasis and/or ion balance maintenance

For both CaOx crystals and cystoliths the most prevalent hypothesis for their function, which is corroborated by several results, concerned the regulation, sequestration or excretion of Ca ions and/or ion balance maintenance (for more recent reviews see He *et al.*, 2013; Paiva, 2019). The presence of crystals in the bundle sheaths may be related to these functions. It has been hypothesised that their presence near phloem or in cells of this tissue is the result of the need to control cytosolic calcium levels that would prevent the transport of photoassimilates (Paiva & Machado, 2005; Paiva, 2019). Moreover the production of insoluble CaOx in the bundle sheaths (Fig. 1d) could create the necessary gradient for the translocation of oxalate between roots and leaves.

Release of CO_2 and alarm photosynthesis

It has been suggested that cystoliths may promote photosynthesis by enhancing the supply of photosynthetic parenchyma cells with CO_2 (Setoguchi *et al.*, 1989; Sugimura *et al.*, 1999). Likewise, Loewus (1999) and Franceschi (1987) proposed a pathway

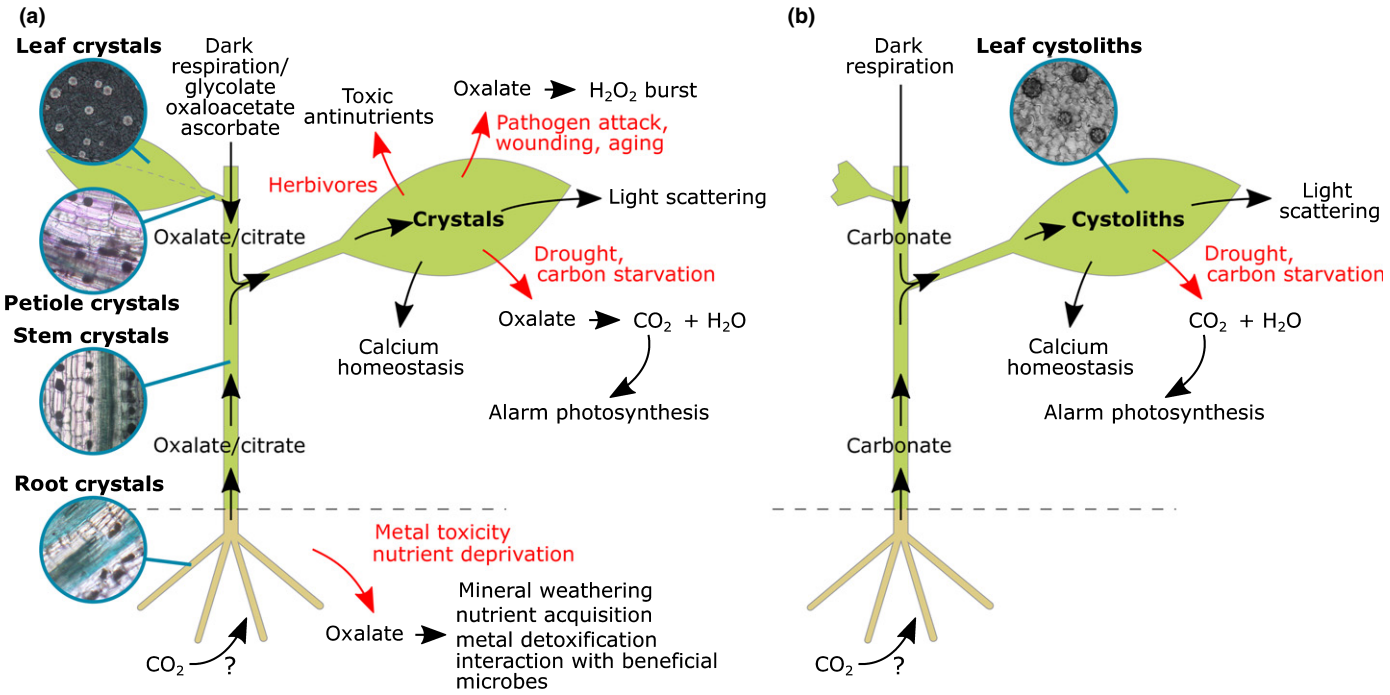

Fig. 3 Simplified schemes showing the main functions of carbon–calcium inclusions in plants. (a) Possible involvement of calcium oxalate (CaOx) crystals in a number of protective and/or defensive functions against multiple stress factors (in red). Round figure inserts in the left part of the figure show hand-cut paradermal sections of the different organs of pigweed stained with toluidine blue O and observed under bright-field optics. The upper left insert shows a cleared leaf of pigweed observed between crossed polarisers. Bright spots are CaOx crystals. Flower CaOx crystals are not included in diagram. These inserts show the whole plant oxalate pool and the CaOx continuum. Citrate, which is a known inhibitor of oxalate precipitation, might help oxalate mobilisation among organs and/or oxalate synthesis in leaves. (b) Possible functions of cystoliths.

involving oxalate decarboxylation as a probable CO_2 source for photosynthesis. Recent evidence from *Amaranthus hybridus* showed that CaOx crystals are dynamically degraded during the day or as a response to stress factors like abscisic acid treatment, drought or carbon starvation (Tooulakou *et al.*, 2016, 2018). In spite of the significant decrease of crystal size, soluble oxalate content was very low or not detectable. This result, together with the increase in oxalate oxidase and catalase activities, showed that oxalate was converted to CO_2. These experiments were combined with metabolomics and chlorophyll fluorescence measurements showing that photosynthetic metabolism was active, in spite of the closed stomata or the absence of CO_2 in the atmosphere (Tooulakou *et al.*, 2016, 2018). All evidence suggests that the released CO_2 is assimilated by a low rate photosynthesis called 'alarm photosynthesis' (Tooulakou *et al.*, 2016). Similar results were also obtained from other species of different functional groups (C_3, C_4 or CAM) under drought conditions (Tooulakou *et al.*, 2016). In a more recent paper, Giannopoulos *et al.* (2019) showed that the cystoliths of *Parietaria judaica* can function in the same manner as the CaOx crystals. The size of cystoliths was reduced under carbon starvation or ABA treatments, whereas it was restored by xylem-fed bicarbonate. Moreover, chlorophyll fluorescence imaging under controlled air composition *in situ* showed that cystolith carbon can be photosynthetically assimilated (Giannopoulos *et al.*, 2019). Thus, CCaIs may function as carbon pools providing CO_2 to photosynthetic cells under drought conditions, when stomata are closed. Under these conditions, carbon acquisition from the atmosphere can become very expensive as well as hazardous to survival in terms of water loss. Thus, alarm photosynthesis supports a low photosynthetic rate, aiming at the maintenance and photoprotection of the photosynthetic apparatus rather than a substantial carbon gain. It is expected however that this function has a significant contribution in growth processes in plants bearing large amounts of CCaIs (such as cacti; Table S1).

The release of water molecules in parallel with the release of CO_2

During dissolution of $CaCO_3$ (Eqn 5), and the dissolution/decomposition of CaOx (Eqn 4) several water molecules are released. Moreover, both inclusions represent hydrated phases and additional molecules of water are released during the dilution of CCaIs. Amorphous $CaCO_3$ contains one molecule of water per $CaCO_3$ (Addadi *et al.*, 2003). CaOx in crystal cells is in either one of two forms: monohydrate $CaC_2O_4{\cdot}H_2O$ (whewellite) or dihydrate $CaC_2O_4{\cdot}(2+x)H_2O$ (weddellite). The dihydrate form has pores, which can accommodate up to 2.5 additional moles of mobile water (zeolitic water) per mole of CaOx (Frey-Wyssling, 1981). Thus, the dissolution of $CaCO_3$ gives two water molecules per CO_2 produced; the dissolution/decomposition of monohydrate CaOx produces two water molecules per two CO_2, and the dissolution/decomposition of dihydrate CaOx up to 6.5 water molecules per two molecules of CO_2. Under drought conditions and closed stomata, the released H_2O may directly replace part of the 'minimum cuticular water losses' (Schuster *et al.*, 2017), supporting a deceptively 'small replenishment' given how 'expensive' and crucial for survival H_2O can become under these conditions. This function may be significant in plants having low cuticular permeability and large amounts of CCaIs, such as cacti. CaOx crystals, especially the dihydrate form, are implied as a possible source of water in the lichens (Wadsten & Moberg, 1985). Zeolitic water can become available for algal cells that are in close proximity to CaOx crystals. During hot days, water is released as vapour, which can be trapped by algal cells in order to maintain a basal photosynthetic activity (Clark *et al.*, 2001). Wadsten & Moberg (1985) reported that lichens from humid climates produced only CaOx monohydrate, while lichens from drier climates contained both forms. Additionally, Cactaceae members of the Cereoideae subfamily deposit only the dihydrate form (Monje & Baran, 2002; Table S1).

Modulation of the light microenvironment

Among the various functions attributed to the CCals is the idea that they may significantly improve the light microenvironment within leaves (Horner *et al.*, 2012). Two recent elegant studies using microscale modulated fluorometry demonstrated that both CaOx crystals and cystoliths are directly involved in light scattering, reducing the steep light gradient and thus enabling the leaf to use the incoming light flux more efficiently (Gal *et al.*, 2012; Pierantoni *et al.*, 2017). CaOx crystals located in the bundle sheaths and their extensions (Fig. 1d) may also play an optical role, as these extensions transfer light into deep internal layers of the mesophyll (Karabourniotis *et al.*, 2000). More experimental effort is needed in this field.

Differences between CCaIs

An obvious difference between cystoliths and CaOx is that their dissolution produces different carbonaceous compounds that is CO_2 (Eqn 5) and oxalate anions (Eqn 1), respectively. This is very important because oxalate is a multifunctional metabolite involved in a number of essential functions (Palmieri *et al.*, 2019; to be described later). This difference may explain the broader spread of CaOx along plant families compared with the limited spread of cystoliths. CaOx may serve a broad spectrum of functions, apart from those served by the cystoliths. Hence, it is not unexpected that there are species such as *Morus alba* and *Ficus* sp. that bear both types of CCaIs, that is cystoliths in the leaf lamina and CaOx crystals in the bundle sheaths (Katayama *et al.*, 2007). Moreover CaOx crystals may be localised in any organ or tissue within the plant body. They are observed in leaves, roots, stems, fruits, and seeds, and within epidermal, parenchyma, and vascular tissues (Franceschi & Horner, 1980). Information concerning calcium carbonate is limited, however it seems that they are formed mainly in photosynthetic organs.

Advantages of bearing CaOx crystals related to multifunctionality of oxalate

Root–soil interactions

Oxalic acid is involved in many processes operating in the rhizosphere, including metal detoxification (Ma *et al.*, 2001) and

nutrient acquisition and mineral weathering (Igamberdiev & Lea, 2006). For example in acid soils, the availability of aluminium and the probable appearance of its toxicity are high. Al tends to bond with phosphorus in a less available and insoluble form in these soils, thereby creating P deficiency in plant organs. Some organic acids, including oxalate are secreted by the roots and chelate Al in a nontoxic complex, preventing Al toxicity (Bojórquez-Quintal *et al.*, 2017).

Thus the CaOx crystals in roots (Kausch & Horner, 1984; Fig. 3) may constitute a major pool of oxalate related to the above processes. Moreover, it has been shown that roots are able to decompose CaOx crystals and metabolise oxalate (Kausch & Horner, 1984; Franceschi, 1989; Horner *et al.*, 2000). In support of this, Choi *et al.* (2007) found that *Panax ginseng* roots grown in low levels of phosphorus in mountain soils possessed more CaOx crystals compared with field-cultivated ginseng, as an acclimation mechanism to phosphorus deprivation. It is also interesting that ABA, which induces the decomposition of leaf CCaIs (described earlier), also induces the secretion of oxalate of buckwheat roots (Ma *et al.*, 2001). Secretion of oxalate from roots may also play a significant role in plant selection for beneficial endophytes, while avoiding pathogenic bacteria from the complex soil bacterial communities. Moreover, oxalotrophic properties among endophytic bacteria are required to ensure colonisation and transmission within host plants (Kost *et al.*, 2014). Oxalotrophy was reported to be associated only with plant-beneficial *Burkholderia phytofirmans*, while pathogenic species of the genus are not able to use oxalate (Kost *et al.*, 2014). The discovery of vertically transmitted endosymbiosis between *Oxalis* species and nitrogen-fixing oxalotrophic bacteria of the genus *Bacillus*, suggests unexpected ways in which geophytes might avoid nitrogen deficiency (Jooste *et al.*, 2019). According to these authors, three common nitrogen-fixing *Bacillus* spp. have known oxalotrophic properties and appear to be housed inside crystal cells within the plant body and seeds.

Modification of cell wall properties – programmed cell death (PCD)

According to Eqn 3, CaOx decomposition produces one molecule of H_2O_2 per oxalate degraded. Thus, in the absence of catalase activity (Eqn 4), oxalate may provide part of the H_2O_2 that participates in a number of vital functions such as lignification and the induction of PCD (Smirnoff & Arnaud, 2019). Oxalic acid is involved in defence reactions of plant tissues against pathogens through production of H_2O_2 and germin-like oxalate oxidase activity increases in response to pathogen attack (Lane, 2002; Ceita *et al.*, 2007), wounding (Le Deunff *et al.*, 2004) and aging (Davoine *et al.*, 2001). Thus, CaOx could represent a pool of oxalate that is able to provide H_2O_2 either for PCD or cell wall strengthening with lignin (Caliskan & Cuming, 2002). Furthermore, there are indications that degradation of CaOx crystals is implicated in PCD during lysigenous aerenchyma formation in *Typha angustifolia* (Du *et al.*, 2018) and during breakdown of the hypodermal stomium and adjacent connective tissue in anthers of *Capsicum annuum* (Horner & Wagner, 1992). Moreover, in *Theobroma cacao*, H_2O_2 production and PCD is triggered by *Moniliophthora perniciosa* infection, likely involving CaOx crystal accumulation and subsequent degradation through activation of an oxalate oxidase gene expression (Ceita *et al.*, 2007; Dias *et al.*, 2011).

Defence against herbivores

Oxalates, as toxic substances, take part in the plant defensive system as chemical weapons. They act as antinutrients, affecting Ca and Mg metabolism and reacting with proteins to form complexes that have an inhibitory effect in peptic digestion by vertebrates (Massey *et al.*, 2001; Thakur *et al.*, 2019). CaOx crystals have been shown to act as a physical deterrent or injury factor (Nakata, 2012). Large needle-shaped crystals can act as a deterrent against larger herbivores (Ruiz *et al.*, 2002) or as an injury factor against larvae (Konno *et al.*, 2014), whereas the smaller nonneedle-shaped prismatic crystals can act as a deterrent against chewing caterpillar larvae such as the beet armyworm, *Spodoptera exigua*. These smaller prismatic crystals act as a physical abrasive that causes damage to the caterpillar mandibles (teeth) during feeding (Korth *et al.*, 2006; Park *et al.*, 2009). There are, however, studies showing no involvement of CaOx in the defence against herbivores (Nagaoka *et al.*, 2010 and included references). These studies clearly suggest that plants have evolved protective mechanisms to affect herbivory in certain cases and not in others, depending on the herbivore (i.e. sucking vs chewing insects), type and location of crystals associated with a particular plant organ, and presence of toxic compounds associated with the protoplasm of cells with or without crystals.

Occurrence of CCaIs must be considered on a holistic basis

CCaIs represent multifunctional tools that are essential especially under stress conditions. Both parts of these inclusions serve vital functions. The Ca part controls the levels of cytosolic calcium and immobilises the excess quantities of this element, taking into account that photosynthetic organisms do not have an excretory system. The carbonaceous part serves a number of protective and defensive functions, depending on the type of the CCaI (Fig. 3a,b). This intelligent combination seems to justify the widespread appearance of CCaIs in photosynthetic organisms. Under this area, functions that seem to be incompatible have to coordinate targeting on the best performance of the organism facing environmental stresses. For example the function of inclusions as carbon or water sources prerequisites the occurrence of a physiological mechanism for the simultaneous fine control of calcium levels. Dissolution of CaOx crystals or cystoliths will cause a massive release of Ca^{2+} ions in the vacuole of the idioblasts. In this case, free calcium would tend to enter the cytosol and disturb the very delicate balance of calcium homeostasis. There are several data suggesting that at least the CaOx crystal cells possess the suitable structural and biochemical characteristics to buffer this massive release of calcium. Concerning structure, a typical characteristic of the crystal cells is the presence of a dense endoplasmic reticulum, which is an important site of Ca^{2+} storage and release. Concerning the biochemical regulatory

mechanisms, several calcium-binding proteins, such as calsequestrin (Franceschi *et al.*, 1993; Li *et al.*, 2003) and calreticulin (Nakata *et al.*, 2003), are localised in the endoplasmic reticulum of the crystal cells. Calsequestrin is a high-capacity (each protein molecule binds up to 50 Ca molecules) calcium-binding protein (Franceschi *et al.*, 1993). Calreticulin affects intracellular Ca^{2+} homoeostasis by modulation of endoplasmic reticulum Ca^{2+} storage and transport (Michalak *et al.*, 1999; Nakata *et al.*, 2003). Recently it was found that CaOx crystals in ovary cells are associated with arabinogalactan proteins that could act as calcium binding and storage molecules (Leszczuk *et al.*, 2019). Moreover several substances accumulate within the vacuoles of crystal cells, some of them with strong Ca-binding capacity. For example raphides are surrounded by a mucilage consisting mainly of complex polysaccharides (Kausch & Horner, 1984; Wang *et al.*, 1994; Webb *et al.*, 1995). It was suggested that the above mentioned proteins and the other Ca-binding substances have a potential role in mediating exchange between the cytoplasm and vacuole of the crystal cells, controlling the passage of calcium (Webb, 1999). Recent findings have shown that, in *Medicago lupulina* plants, a high external calcium concentration causes an accumulation of CaOx crystals, as well as an induction of the genes encoding Ca transporters and calcium-binding proteins such as calreticulin (Zhang *et al.*, 2019). This indicates that CaOx-bearing plants have the ability to control strong calcium fluxes. Concerning lithocysts, the available data are limited; polysaccharides could play the regulatory role of calcium binding during cystolith dissolution. Mucilage-containing epidermal cells are localised near lithocysts of *Morus alba* leaves (Katayama *et al.*, 2007).

A critical question concerning CaOx is why these inclusions are localised in almost every organ of a plant (Fig. 3a). There are two probable explanations: (1) There is a transfer of oxalate (or its precursors) and Ca from one organ to another; and (2) Due to the multifunctionality of oxalates, CaOx in each organ may serve a different function. Concerning the first explanation, according to recent evidence, oxalate from the root is transferred to the leaves via xylem and could take part in the construction of leaf CaOx (Tooulakou *et al.*, 2016). Moreover, citrate translocated from stems is used in the isocitrate pathway as a precursor for oxalate synthesis in *Rumex* leaves (Miyagi *et al.*, 2013.) For the second explanation, oxalic acid produced in the root can take part in nutrient acquisition, metal detoxification, mineral weathering and selection of beneficial bacterial populations, whereas oxalic acid in the leaves can take part in alarm photosynthesis. However, both root and leaf oxalate can take part in defence reactions upon pathogen and/or herbivore attack (Fig. 3a). A key enzyme for this dual behaviour is catalase. High activity of catalase will result in H_2O_2 cleavage and photosynthetic assimilation of CO_2, whereas inhibition of this enzyme will result in H_2O_2 burst and PCD. We speculate that CaOx crystals of the belowground and aboveground organs of plants (Fig. 3a) create a continuum constituting a single oxalate pool that could be used to confront a range of different biotic and abiotic stresses, depending on the particular organ and the particular stimulus (Fig. 3a).

Root-borne carbon in the form of oxalate could be formed either by refixation of respiratory CO_2 or by fixation of soil CO_2/HCO_3. In the first case, oxalate can be synthesised from glyoxylate (see section on Occurrence of CCaIs in photosynthetic organisms is an ancient trait) whereas, in the second, by the oxidative cleavage of oxaloacetic acid (the product of PEP carboxylase reaction; see Rivera & Smith, 1979; see also section on Occurrence of CCaIs in photosynthetic organisms is an ancient trait section). It is probable, therefore, that PEP carboxylase is implicated in the synthesis of root oxalate by fixation of respiratory or soil CO_2. Root oxalate could also be derived from mycorrhizal fungi, which use bicarbonate as a carbon source for oxalate formation (Lapeyrie, 1988).

Conclusion and future perspectives

The body of sophisticated literature dealing with CCals continues to increase, showing their importance in the lives of many photosynthetic organisms from the photosynthetic bacteria to the angiosperms. Multiple functions of CaOx crystals provide tolerance against a combination of abiotic (drought, nutrient deprivation, metal toxicity) and biotic (pathogens, herbivore) stress factors. Recent findings on the implication of these intriguing structures in alarm photosynthesis and endosymbiosis with beneficial bacteria could provide valuable pillars for development of innovative tools regarding plant stress tolerance. Research in this direction has become of great significance, as climate change scenarios predict an increase in extreme environmental conditions in many parts of the world, reducing both ecosystem and agricultural productivities. Furthermore, as part of the phytomineralisation process, CaOx crystals represent a considerable carbon sink at the ecosystem (and global) level. Oxidation of litter oxalate by soil oxalotrophic bacteria gives rise to the oxalate–carbonate pathway leading to $CaCO_3$ accumulation and a local soil pH increase. $CaCO_3$ may then accumulate, modifying soil conditions and sequestering carbon and Ca in an inorganic form with a longer residence time than organic carbon (Cailleau *et al.*, 2014; Turpault *et al.*, 2019). Further research could lead to the exploitation of this biogeochemical process as an important global regulator of soil Ca concentration (Turpault *et al.*, 2019) and of atmospheric CO_2 levels, mitigating the greenhouse effect (Cailleau *et al.*, 2011). It is clear that these possibilities will elicit further research to show how CCals play a significant role in the larger area of biomineralisation.

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Supporting Information

Additional Supporting Information may be found online in the Supporting Information section at the end of the article.

Table S1 Species bearing calcium oxalate (CaOx) from different sites globally.

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