New insights on the functions of carbon-calcium-inclusions in plants

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Abstract
The carbon-calcium-inclusions (CCals) 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 level, and the release of CO2 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 to CaCO3 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 reevaluation 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.

Keywords
biomineralization, calcium oxalate crystals, carbon calcium inclusions, cystoliths, drought stress

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New insights on the functions of carbon-calcium-inclusions in plants

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Summary

The carbon-calcium-inclusions (CCaIs) 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 level, 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 to 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 reevaluation 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.

Keywords biomineralization, calcium oxalate crystals, carbon calcium inclusions, cystoliths, drought stress
Introduction

The occurrence of carbon-calcium-inclusions (CCaIs) has been reported by pioneer anatomists for both plant and animal kingdoms as far back as the seventieth century. Calcium oxalate (CaOx) crystals (Fig. 1b, c, d) and calcium carbonate (CaCO$_3$ lime) cystoliths (Fig. 1a) are common cellular solid deposits in plants and represent biomineralization (Bauer et al., 2011; He et al., 2013). The evolution of biomineralization has been linked to the formation of primitive organisms and the diversity of materials, mechanisms and strategies used by an impressive diversity of biomineralizing life forms. In this regard, Skinner and Jahren (2003) raised the question “why biomineralize?” and suggested the answer lies on the two main adaptive advantages afforded by this process: a. Physical, by the construction of skeletal components and b. 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 biomineralization in which case the storage system has to be dynamic so as to have the ability to offer back to the organism the stored materials. Of course this generalization is not the rule in every case; for example bones serve as a reservoir for and source of calcium for critical metabolic needs in mammals (Ross et al., 2011). On the other hand, some biominerals, e.g., 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). In contrast, CaOx is the most prevalent and widespread biomineral in plants (Franchschi and Horner, 1980; Horner and Wagner, 1995), with reduced occurrence in other organisms. It seems therefore that CaOx may represent a more prevalent storage system for plants compared to CaCO$_3$.

Taking into account 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 summarize 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 reevaluation 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.
The 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 and 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 about 50% of biominerals (Weiner and Dove, 2003).

Concerning the carbonaceous parts of CCaIs, carbonate can be of both environmental and biological origin, 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 and Eprintsev 2016 and Cai et al. (2018). The three most significant pathways reported are:

1. from the oxidation of glyoxylate. Glyoxylate can be formed during photosynthesis/photorespiration as a product of glycolate oxidase reaction (glycolate is oxidized to oxalate) or of isocitrate lyase reaction (isocitrate is converted to glyoxylate and succinate).
2. from the oxidative C2/C3 cleavage of L-ascorbic acid via several steps. An intermediate metabolite of this path is the dehydroascorbic acid (Zhang et al., 2019)
3. from the oxidative cleavage of oxaloacetate (derived either from the PEP carboxylase reaction or from citrate), the reaction catalyzed 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 non-photosynthetic organs of plants (i.e., roots; Horner et al., 2000, see also Section VIII) and at least in spinach, all the putative genes involved are 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 CaCO3 deposition preceded CaOx deposition. Hence, it is not surprising that CaCO3 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 CaCO3 (calcification) is widespread in these primitive unicellular organisms offering adaptive advantages (Castanier et al., 2012). Intracellular...
deposition, contrary to the extracellular one 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, e.g. Cannabaceae, Moraceae, Ulmaceae and Urticaceae 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, where the CaCO$_3$ is deposited over a cellulosic stalk hanging from the cell wall (Bauer et al., 2011, see Fig. 1a). While studies on fossils revealed that CaCO$_3$ deposition by photosynthetic microorganisms dates back to 3.3 bya (Tice and Lowe, 2004), data for CaOx deposition are limited to only extant taxa as CaOx is not preserved in extinct taxa so 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 and 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 glycolate oxidase reaction due to the appearance of photorespiration (Igamberdiev and 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; 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
The 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 of rain forests have leaves with CaOx crystals. This percentage is increased in montane forests (76-86%) and even higher in xerophytes and poikilohydric organisms such as lichens (Supporting information, 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 about 100 Kg CaOx (Garvie, 2006). Moreover, among different desert species (psammophytes) the drought-resistant plants bear more CaOx crystals compared to the 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 percent 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 affecting the production of crystals such as the soil calcium availability and the 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 to those growing in higher rainfall areas. There are also links between the accumulation of CaOx crystals in tree rings and seasonal drought fluctuations (Gourlay and Grime, 1994).

Some similar characteristics of CCaIs

1. Both CCaIs are calcium and carbon pools in a solid form which are metabolically and osmotically inactive without obstructing cell functions.
2. The decomposition of both CCaIs can produce Ca ions, $CO_2$ and $H_2O$. In the case of CaOx, crystal dissolution is a prerequisite for further decomposition.
CaOx dissolution:

\[ \text{CaOx} \rightarrow \text{Ca}^{2+} + \text{oxalate ions} + x\text{H}_2\text{O}^* \text{ (reaction 1)}. \]

CaOx decomposition:

- oxalate + O\(_2\) + 2 H\(^+\) → 2 CO\(_2\) + H\(_2\)O\(_2\) (oxalate oxidase) \text{ (reaction 2)}
- 2 H\(_2\)O\(_2\) → 2 H\(_2\)O + O\(_2\) (catalase) \text{ (reaction 3)}

Sum of reactions 1, 2 and 3

\[ \text{CaOx} \rightarrow \text{Ca}^{2+} + 2 \text{CO}_2 + \text{O}_2 + x\text{H}_2\text{O}^{**} \text{ (reaction 4)**} \]

Cystolith dissolution/decomposition:

\[ \text{CaCO}_3 + 2 \text{H}^+ \rightarrow \text{Ca}^{2+} + \text{CO}_2 + \text{H}_2\text{O} \text{ (reaction 5)} \]

* In reaction 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 reaction 4 water molecules may be zeolitic, embedded in the crystal or produced by the reaction 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 below Webb, 1999; Leszczuk et al., 2019) or the presence of substances which will form soluble complexes with either calcium or oxalate ions, such as citric acid and magnesium is a possible mechanism. Note that decomposition reactions (2 and 5) of both CCaIs require the presence of protons and in the case of reaction 2 oxygen.

3. Both CCaIs are dynamic storage systems which 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 the 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 have been 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 the lithocysts or the crystal cells are strategically placed either among the photosynthesizing parenchyma or in the bundle sheaths and the sequestered carbon in these structures is considerable (Fig. 1; Fig. 3).

5. Measurements of $\delta^{13}$C showed that at least in some plant species (Rivera and Smith, 1979; Toulakou et al., 2016) and lichens (Beazley et al., 2016), 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 the 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 hypothesized 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, 2019; Paiva and Machado, 2005). 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.

**The 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 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; Tooulakou et al., 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 CO₂. 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 CO₂ in the atmosphere (Tooulakou et al., 2016; Tooulakou et al., 2018). All evidence suggests that the released CO₂ 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 (C₃, C₄ 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 CO₂ 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 CO₂**

During dissolution of CaCO₃ (reaction 5), and the dissolution/decomposition of CaOx (reaction 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 CaCO₃ contains one molecule of water per CaCO₃ (Addadi et al., 2003). The CaOx in crystal cells is either one of two forms: monohydrate CaC₂O₄·H₂O (whewellite) or dihydrate CaC₂O₄·(2+x) H₂O (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 CaCO₃ gives two water molecules per CO₂ 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$_2$O may directly replace a part of the “minimum cuticular water losses” (Schuster et al., 2017), supporting a deceptively “small replenishment” given how “expensive” and crucial for survival can H$_2$O become under these conditions. This function may be significant in plants having low cuticular permeability and large amounts of CCaIs, such as cacti. The CaOx crystals, especially the dihydrate form, are implied as a possible source of water in the lichens (Wadsten and Moberg, 1985). The zeolitic water can become available for the algal cells which are in close proximity to CaOx crystals. During hot days, the water is released as vapor which can be trapped by the algal cells in order to maintain a basal photosynthetic activity (Clark et al., 2001). Wadsten and 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 and Baran, 2002; Supportive Information: Table S1).

The modulation of the light microenvironment

Among the various functions attributed to the CCaIs is the idea they may significantly improve the light microenvironment within leaves (Horner, 2012). Two recent elegant studies using micro-scale 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, since 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 i.e. CO$_2$ (reaction 5) and oxalate anions (reaction 1), respectively. This is very important because oxalate is a multifunctional metabolite involved in a number of essential functions (Palmieri et al., 2019, see also next chapters). This difference may explain the broader spread of CaOx along plant families compared to 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 i.e. cystoliths in the leaf lamina and CaOx crystals in the bundle sheaths (Katayama *et al.*, 2007). Moreover CaOx crystals may be localized 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 and Horner, 1980). The information concerning calcium carbonate is limited; however it seems that they are formed mainly in photosynthetic organs.

**Advantages of bearing CaOx crystals related to the 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 and Lea, 2006). For example in acid soils, the availability of aluminum 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 non-toxic complex, preventing the Al toxicity (Bojórquez-Quintal *et al.*, 2017). Thus the CaOx crystals in roots (Kausch and Horner, 1984; Fig. 3) may constitute a major pool of oxalate related to the above processes. Moreover, it has been shown that the roots are able to decompose CaOx crystals and metabolize oxalate (Franceschi, 1989; Horner *et al.*, 2000; Kausch and Horner, 1984). 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 to field-cultivated ginseng, as an acclimation mechanism to phosphorus deprivation. It is also interesting that ABA which induces the decomposition of leaf CCaIs (see previously) also induces the secretion of oxalate of buckwheat roots (Ma *et al.*, 2001). The 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, the oxalotrophic properties among endophytic bacteria are required to ensure colonization 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 reaction 3, CaOx decomposition produces one molecule of H$_2$O$_2$ per oxalate degraded. Thus, in the absence of catalase activity (reaction 4) oxalate may provide part of the H$_2$O$_2$ which participates in a number of vital functions such as lignification and the induction of PCD (Smirnoff and Arnaud, 2019). Oxalic acid is involved in defense reactions of plant tissues against pathogens through the production of H$_2$O$_2$ and the 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 which is able to provide H$_2$O$_2$ either for PCD or cell wall strengthening with lignin (Caliskan and Cuming, 2002). Furthermore, there are indications the degradation of CaOx crystals is implicated in PCD during the lysigenous aerenchyma formation in *Typha angustifolia* (Du *et al*., 2018) and during the breakdown of the hypodermal stomium and adjacent connective tissue in the anthers of *Capsicum annuum* (Horner and Wagner, 1992). Moreover, in *Theobroma cacao* H$_2$O$_2$ 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).

**Defense against herbivores**

Oxalates, as toxic substances, take part in the plant defensive system as chemical weapons. They act as anti-nutrients, affecting Ca and Mg metabolism and reacting with proteins to form complexes which 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 non-needle 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 defense against herbivores (Nagaoka et al., 2010 and included references). All above 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.

The occurrence of CCaIs must be considered on a holistic basis

The CCaIs represent multifunctional tools which are essential especially under stress conditions. Both parts of these inclusions serve vital functions. The Ca part controls the levels of cytosolic calcium and immobilizes the excess quantities of this element, taking into account that the 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 prism, functions which 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. The 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 posses 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 localized 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). Calreticuline 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 which could act as calcium binding and storage molecules (Leszcuk 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 and 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 the potential role in mediating exchange between the cytoplasm and the vacuole of the crystal cells, controlling the passage of calcium (Webb, 1999). Recent findings showed 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 localized near lithocysts of Morus alba leaves (Katayama et al., 2007).

A critical question concerning CaOx is why these inclusions are localized 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 multi-functionality of oxalates, CaOx in each organ may serve a different function. Concerning the first explanation, according to recent evidence oxalate from root is transferred to the leaves via xylem and could take part to the construction of leaf CaOx (Tooulakou et al., 2016). Moreover, citrate translocated from stems is used in isocitrate pathway as a precursor for oxalate synthesis in Rumex leaves (Miyagi et al., 2013.) Concerning the second one, 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 defense reactions upon pathogen and/or herbivore attack (Fig. 3a). A key enzyme for this dual behavior is catalase. High activity of catalase will result in H₂O₂ cleavage and photosynthetic assimilation of CO₂, whereas inhibition of this enzyme will result in H₂O₂ 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 which 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 CO₂ or by fixation of soil CO₂/HCO₃. In the first case oxalate can be synthesized from glyoxylate (see
Section II) whereas in the second by the oxidative cleavage of oxaloacetic acid (the product of PEP carboxylase reaction, see Rivera and Smith 1979, see also Section II). It is probable, therefore, that PEP carboxylase is implicated in the synthesis of root oxalate by the 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. The multiple functions of CaOx crystals provide tolerance against a combination of abiotic (drought, nutrient deprivation, metal toxicity) and biotic (pathogens, herbivore) stress factors. The recent findings of the implication of these intriguing structures in alarm photosynthesis and the endosymbiosis with beneficial bacteria could provide valuable pillars for the development of innovative tools regarding plant stress tolerance. Research towards this direction has become of great significance, as climate change scenarios predict increment of extreme environmental conditions in many parts of the world reducing both ecosystem and agricultural productivity. Furthermore, as part of the phytomineralization process, CaOx crystals represent a considerable carbon sink at the ecosystem (and global) level. The 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 the 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 biomineralization.
References


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Franceschi VR. 1989. Calcium oxalate formation is a rapid and reversible process in *Lemna minor* L. *Protoplasma* 148: 130–137.


Garvie LAJ. 2006. Decay of cacti and carbon cycling. *Naturwissenschaften* 93:114-118


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Lapeyrie F. 1988. Oxalate synthesis from soil bicarbonate by the mycorrhizal fungus *Paxillus involutus*. *Plant and Soil* **110**: 3-8


Leszczuk A, Wydrych J, Szczuka E. 2019. The occurrence of calcium oxalate crystals and distribution of arabinogalactan proteins (AGPs) in ovary cells during *Fragaria x ananassa* (Duch.) development *Journal of Plant Growth Regulation* **38**: 1028-1036.


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Supporting Information

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

Figure 1. a. *Ficus elastica* (Moraceae) leaf lithocyst. The CaCO$_3$ has been dissolved during the fixation of the sample and the cellulosic stalk of the cystolith is obvious (black arrow). Scale 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. Scale bar = 50 μm. c. *Uncaria canescens* (Rubiaceae) leaf mesophyll idioblasts (white arrow) each containing crystal sand and a druse (light blue arrow). d. *Fagus* sp. (Fagaceae) leaf showing prisms (yellow arrows) associated with veins (v) and in mesophyll. Scale bar = 100 μm. b. observed between partially and c.,d, completely crossed polarizers. V, veins. Scale bar = 50 μm.

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

Figure 3. Simplified schemes showing the main functions of carbon-calcium-inclusions in plants. a. The 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 with bright-field optics. The upper left insert shows a cleared leaf of pigweed observed between crossed polarizers. 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 the oxalate mobilization among organs and/or oxalate synthesis in leaves. b. Possible functions of cystoliths.
A scatter plot showing the relationship between species with crystals (%) and mean annual precipitation (mm). The correlation coefficient is $r = -0.79$, with $p < 0.01$. The data points are scattered along a downward-sloping line, indicating a negative correlation.