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RESEARCH PAPER

Calcium oxalate crystal production and density at different phenological stages of soybean plants (*Glycine max* L.) from the southeast of the Pampean Plain, Argentina

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ABSTRACT

- *Glycine max* L. (soybean) is one of the major crops of the world. Although the process of biomineralisation has been reported in some organs of soybean, we now report the description and quantification of calcium oxalate crystals in vegetative and reproductive organs of soybean during its life cycle, as they act as an important source of calcium to the soil, once the harvesting is finished.
- Through diaphanisation, cross-sectioning, optical and scanning electron microscopy analysis of the organs, morphology, size and location of the crystals were identified. In addition, crystal density (n° crystals·mm^{-2}) and the input of crystals to soil (n° crystals·ha^{-1}) were calculated.
- Soybean produced prismatic calcium oxalate crystals in vegetative and reproductive organs, generally associated with vascular bundles, resulting in a potencial transfer to the soil of 81.4 x 10^{7} crystals·ha^{-1} throughout its life cycle. Pods were the organs with higher calcium oxalate crystal production (1112.7 ± 384.6 crystals·mm^{-2}), but with the smaller size (12.3 ± 2.1 μm long). However, cotyledons were the organs that produce the larger crystals (21.3 ± 3.5 μm long), but in lesser amounts (150.9 ± 64.4 crystals·mm^{-2}). In leaves, although crystal size did not differ from vegetative to reproductive stage (14.5 ± 4.2 and 14.5 ± 4 μm in length, respectively), the crystal density increased (293.2 and 409 crystals·mm^{-2}, respectively).
- These results will contribute to knowledge of the amount of calcium oxalate crystals involved in the process of Ca recycling through cultivated vegetation in fields from humid plains at medium latitudes, which therefore have biological, botanical, biogeochemical and pedological relevance.

INTRODUCTION

Biomineralisation is a well-known process in plants (Franceschi & Horner 1980; Piperno 2006). In general, the term 'biomineralisation' comprises mineral or amorphous structures of varied chemical composition, generated by metabolic activity of different organisms (Coe *et al.* 2014). In plants, the most common biomineralisations are silicophytoliths (hydrated amorphous silica) and calciphytoliths, with calcium carbonates and oxalates as the predominant forms (Metcalfe 1985). These biomineralisations have essential functions for plants: they are involved in bulk calcium regulation, provide tissue rigidity and support, stimulate canopy photosynthesis by improving leaf erectness, promote the growth of plants, reduce vulnerability to insect damage and pathogens, confer resistance to herbivory, alleviate water and various mineral stresses, and ameliorate heavy metal toxicity by immobilising Al, Fe, Mn and Zn (Franceschi & Horner 1980; Epstein 1999; Prychid & Rudall 1999; Molano-Flores 2001; Massey *et al.* 2007). In addition, since phytolith formation is genetically controlled, their morphology and distribution among plant organs and tissues may help in plant taxon identification (Twiss *et al.* 1969; Franceschi & Horner 1980).

The presence of calcium oxalate crystals have been reported in more than 215 plant families (Nakata 2003); being widespread in the Fabaceae (Zindler-Frank 1987). Many studies have reported the presence of prismatic crystals in the Fabaceae (Franceschi & Horner 1980; Duarte & Wolf 2005; Borrelli *et al.* 2009; He *et al.* 2013), especially in species belonging to the Faboideae subfamily (Franceschi & Horner 1980; Zindler-Frank *et al.* 2001; Nakata 2002; Jáuregui-Zúñiga *et al.* 2003; Jáuregui-Zúñiga *et al.* 2005).

Soybean (*Glycine max* L., Fabaceae, Faboideae) is one of the major crops in the world. In Argentina, soybean occupies about 50% of the total cultivated area, exceeding 15 million ha in 2006, and represents the main export product. Hence, by surface occupied and production, soybean is the most important crop, making Argentina one of the leading production countries worldwide (Aizen *et al.* 2009). In particular, the southeast of the Pampean Plain is an important production area. Soils, typic Argiudolls, have evolved from aeolian sediments linked to the latest arid cycle of the late Pleistocene–early

Holocene (Osterrieth & Cionchi 1985). Since about 150 years ago, intense agricultural and horticultural activity developed in the study area, causing a noticeable decrease in the organic matter and clay fraction content of the topsoil, with subsequent loss of structural stability and fertility (Osterrieth & Maggi 1996; Osterrieth *et al.* 1998, 2001). It is therfore neccesary to better understand the biogeochemical cycles of elements, especially those affecting soil stability, to prevent further damage and ensure productivity. In this sense, the importance of biomineralisations in biogeochemical cycles has been reported (Pinilla *et al.* 1993; Conley 2002; Derry *et al.* 2005; Borrelli *et al.* 2009, 2010; Schmitt *et al.* 2013; Benvenuto & Osterrieth 2015). In particular, calcium oxalate crystals are an important source of Ca to the soil solution, since they rapidly disolve in the organic horizons once the plant dies and become part of the soil (Pinilla *et al.* 1993; Borrelli *et al.* 2009). Calcium in its soluble form can re-enter the plant cycle, participate in formation of secondary minerals and/or conform organic–mineral complexes necessary for structural stability of soil aggregates influencing the development of soils (Porta Casanellas *et al.* 1999; Borrelli *et al.* 2009).

Given the economic and nutritional importance of soybean, there are already studies dealing with the presence of calcium oxalate crystals in seeds and leaves (Ilarslan *et al.* 1997, 2001; Cervantes-Martinez *et al.* 2005). However, as during the harvesting process, with the exception of the seed, all other organs remain in the field; hence it is necessary to understand production of the whole plant to estimate the contribution of calcium oxalate crystals of the soybean crop. A recent study reported silicophytolith production during the life cycle of soybean (Benvenuto & Osterrieth 2015), but there is no information about calciphytolith production. The aims of our work are to: (i) describe and quantify the production of calcium oxalate crystals in vegetative and reproductive organs at different growth stages during the life cycle of soybean grown in typic Argiudolls of the southeast of the Pampean Plain; and (ii) estimate the contribution of calcium oxalates to the soil.

MATERIAL AND METHODS

Study area

The southeast of Buenos Aires province (38°12′ S, 57°48′ W; Fig. 1) is part of an ecosystem of meadows that were dominated by native grasslands during the Cenozoic (Darwin 1983). It belongs to the geomorphological unit known as 'Perinange aeolian hills', which comprises a relief of morphologically complex hills, with relative heights of up to 30 m and concave–convex profiles with intermediate straight patches and slopes between 6% and 8% (Osterrieth *et al.* 1998). The hills originated from processes of primary aeolian accumulation, modified later by superficial wash (Osterrieth & Martínez 1993).

Typic Argiudolls are the most representative soils in the area. They originated from aeolian sediments during the latest arid cycle of the late Pleistocene–early Holocene (Osterrieth & Cionchi 1985). They have a mollic epipedon (A horizon) over 30-cm thick and an argillic endopedion (B horizon) of approximately 50 cm. The organic matter content is high, 9.39% in the A horizon, 3.00% in the B horizon and 1.50% in the parent material (loess). The soil texture is silt loam, with the silt (55–70%) and clay (20–30%) fractions being the most representative (Borrelli *et al.* 2008). Soil mineralogy is predominantly composed of light minerals: Ca–Na feldspars (25–30%), vitroclastics (25–30%), quartz (20%), volcanic ashes (10%) and K felspars (5%; Borrelli *et al.* 2009). Heavy minerals, such as pyroxenes, amphiboles, opaques, alterites, epidotes, garnets, tourmalines and zircons, represent only 0.9–4.0% (Borrelli *et al.* 2009).

In this región, the climate is mesothermic and subhumid, with little or no water deficiency (Burgos & Vidal 1951). From December 2012 to March 2013 the measured mean precipitation was 91.91 ± 50.71 mm. During this period mean maximum temperature ranged from 15.2 to 27.3 °C and minimum from 8.7 to 14.4 °C (Meteorological Station from Mar del Plata: 876920-SAZM). Following the standards set by the Soil Survey Staff (1996), the soil temperature regime is mesic, and the regime of humidity is udic.

Collection of plant material

Three samplings were performed in December 2012, February and March 2013 to collect individuals of soybean at the following growth stages: vegetative (S12), plants with trifoliolate leaf on the second node unfolded (Fig. 2A and D); reproductive (S61), plants at the start of flowering (Fig. 2B and E); and maturity (S89), plants with the majority of pods ripe and beans dry and hard (Fig. 2C and F; Munger *et al.* 1997). In each

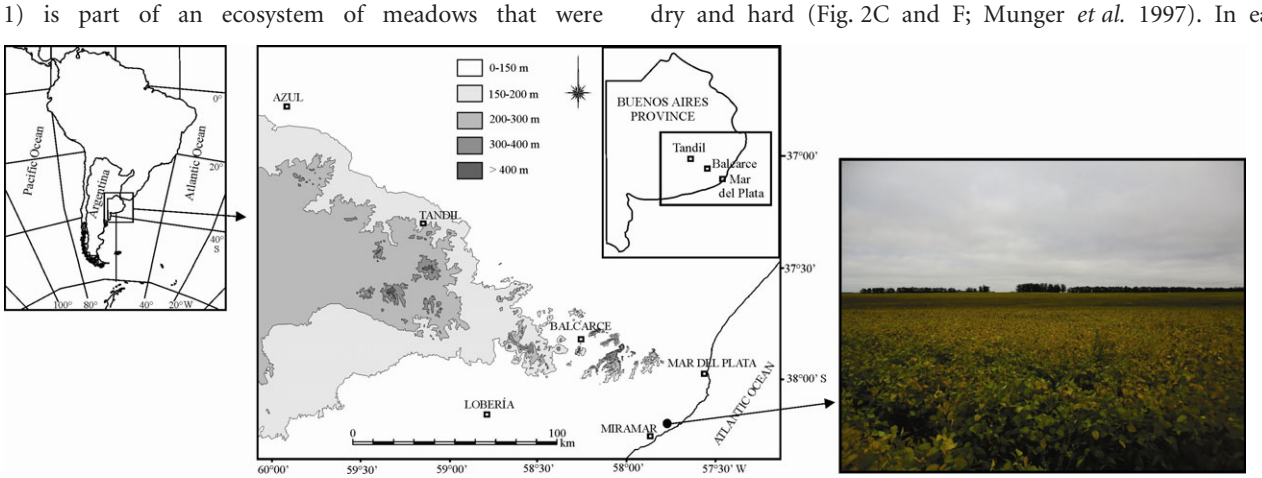

Fig. 1. Location of study area and detail of *Glycine max* field (Benvenuto & Osterrieth 2015).

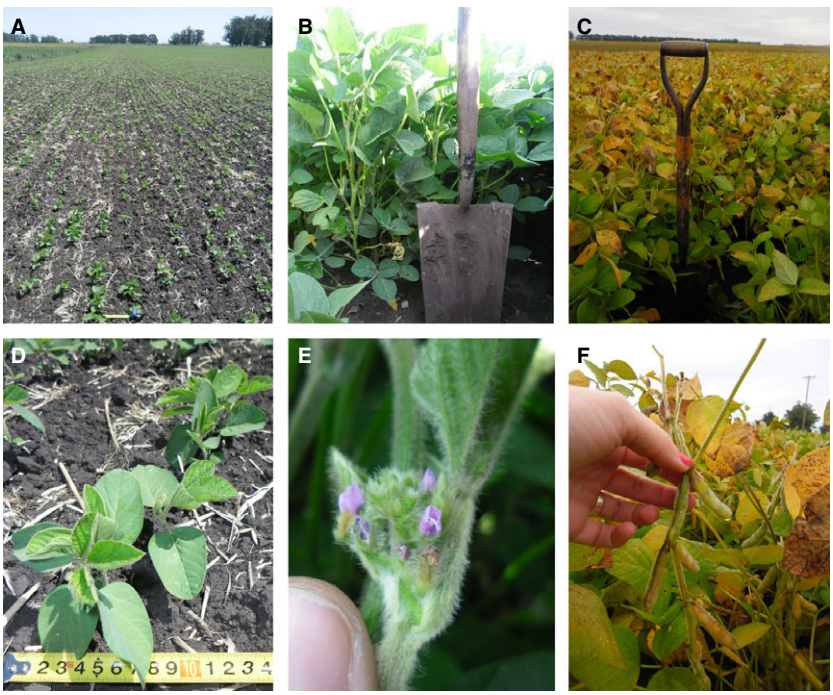

Fig. 2. Different growth stages of *Glycine max* crop. D–F: Detail of principal organs in each stage. S12, vegetative stage (A, D). S61, reproductive stage (B, E). S89, state of mature stage (C, F) (Benvenuto & Osterrieth 2015).

growth stage, nine complete specimens of soybean were randomly collected to describe and quantify the calcium oxalate crystals production. In addition, the biomass of the soybean crop was estimated by extrapolation from three plots of 1 m^2. The specimens were measured (cm) and each organ was carefully isolated so as to obtain cotyledons, leaf primordia, leaves, stems, roots, flowers and pods. Approximately two plants per growth stage were dried and stored in the herbarium of the Soils Geoecology Laboratory (Institute of Coasts Geology and Quaternary, Faculty of Natural Sciences, National University of Mar del Plata, Buenos Aires, Argentina).

Description of calcium oxalate crystals

Three samples from each organ of each soybean growth stage were analysed. All the organs were washed with distilled water and cleaned in an ultrasound bath (Test-Lab, TBC 10 model) in order to remove any adhered material. Afterwards, tissue clarification (Dizeo de Strittmater 1973) and cross-sectioning were applied. The material was mounted with gelatine–glycerine, and calcium oxalate crystals were identified and described with a petrographic (Olimpus BX 51P) and optical microscope (Leitz Wetzlar D35780) at 400× magnification.

Samples of pods were dried, gold-coated and observed using a scanning electron microscope (JEOL JSM-6460 LV; Tokyo, Japan) at the National University of Mar del Plata, Buenos Aires, Argentina.

Quantification of calcium oxalate crystals

Crystal density (no crystals·mm^{-2} organ area) was determined in the clarified samples of all the complete vegetative and reproductive organs after treatment described above. Crystal density was calculated by counting all crystals within an area of 0.196 mm^2. Eight (±1) areas in cotyledons (total area per cotyledon: 1.568 mm^2), three (±1) areas in leaf primordia (total area per leaf primordium: 0.588 mm^2), nine (±2) areas in leaves (total area per leaf: 1.764 mm^2), three (±1) areas in flowers (total area per flower: 0.588 mm^2) and seven (±1) areas in pods (total area per pod: 1.372 mm^2) were counted. Photographs were taken with a Kodak Easy Share CX7530 digital camera.

Estimation of calcium oxalate crystal production and input to the soil from plants

The calcium oxalate crystal production in soybean plants (no crystals·ha^{-1}) was calculated as the product of (i) crystal density per organ, (ii) number of organs per plant, (iii) number of plants in the field (52 plants·m^{-2}).

Data analyses

Means ± SE for calcium oxalate crystal sizes and their density in each organ of soybean from different growth stages were calculated. Differences in the size and density of crystals between organs and growth stages were tested with a Kruskal–Wallis test and a non-parametric multiple comparison test, since normality and homoscedasticity assumptions were not achieved (Zar 1984). In leaves, differences in the size of crystals associated with the mibrid and other vascular bundles were tested with Wilcoxon signed rank test for paired data (Zar 1984).

RESULTS

Morphology and distribution of calcium oxalate crystals

All the crystals observed in soybean were prismatic, but with variations in the organs analysed. In cotyledons, the crystals were rectangular, twinned kinked (Fig. 3A–C). Leaf primordia

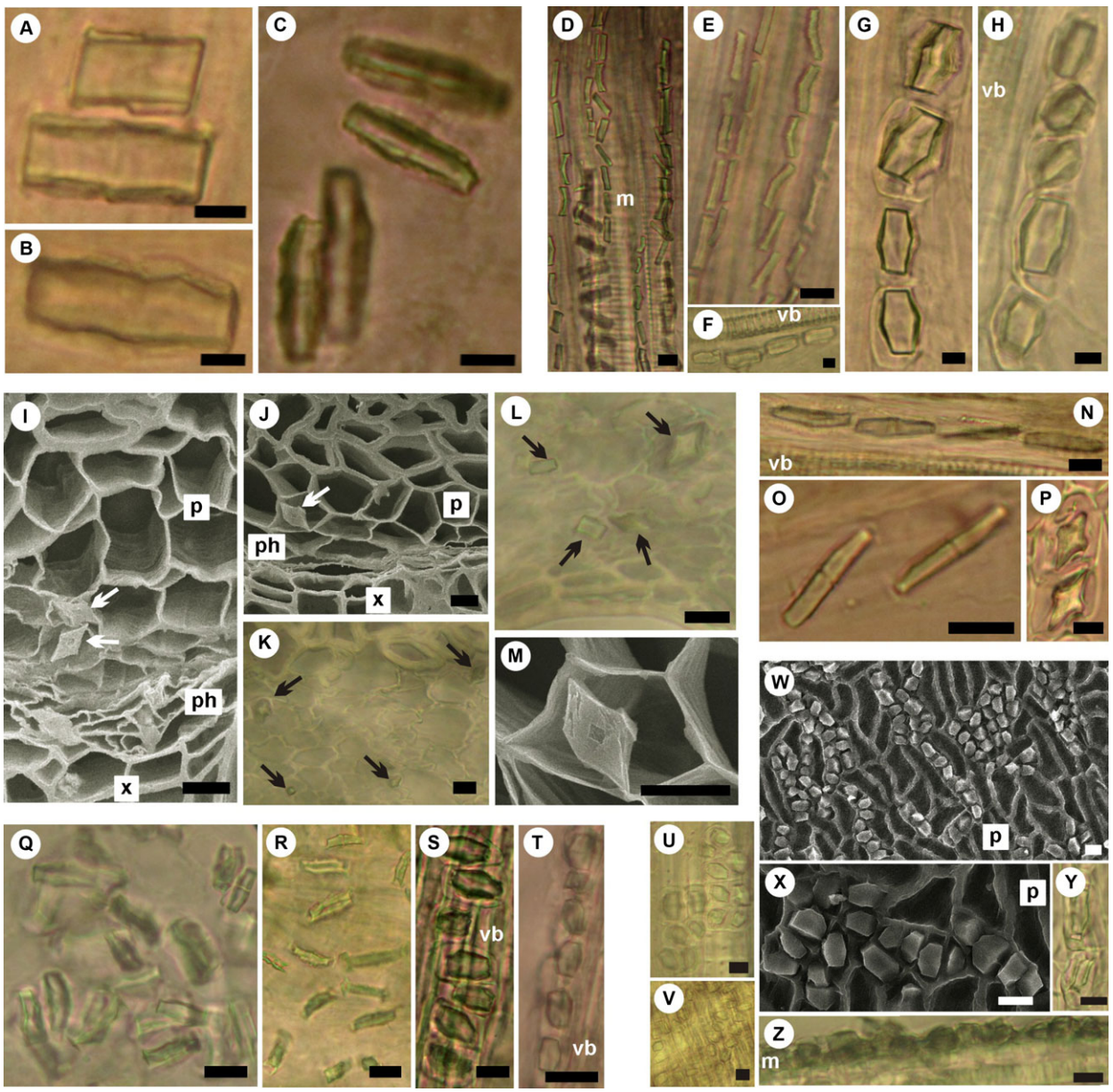

Fig. 3. Prismatic calcium oxalate crystals in vegetative and reproductive organs of soybean plants. A–C: rectangular twinned kinked crystals in mesophyll of cotyledons. D–H: rectangular crystals associated with midrib (D–E) and twinned kinked crystals in vascular bundles of leaf (F–H). I–M: rhomboid crystals developed in phloem of stem. N–P: rectangular twinned crystals associated with vascular bundles (N–O), and rhomboid crystals in the parenchyma of the cortex (P) in roots. Q–T: twinned and rectangular twinned crystals in mesophyll (Q–R) and vascular bundles (S–T) in calyx. U–Z: twinned kinked crystals in parenchyma of mesocarp (U–Y) and along the midrib of carpel in pods. m = mibrid, vb = vascular bundle, p = parenchyma, ph = phloem, x = xylem. Scale bars: 10 μm.

had kinked prismatic crystals. In the midrib of leaves, rectangular crystals were observed (Fig. 3D and E), while in vascular bundles of the leaf blade, the crystals were twinned kinked (Fig. 3F–H). The stems had rhomboidal prismatic crystals (Fig. 3I–M). In roots, rectangular twinned crystals were associated to vascular bundles (Fig. 3N–O), while rhomboidal crystals were observed in the cortex (Fig. 3P). The crystals in the calyx were twinned and rectangular twinned (Fig. 3Q–T), while the pods, showed twinned kinked crystals (Fig. 3U–Z).

The data on size of the crystals in the organs at different growth stages are presented in the Table 1. Cotyledons and roots were the organs with larger calcium oxalate crystals (21.3 and 21 μm in length, respectively), while pods were the organs with smaller prismatic crystals (11.7 μm in length; Fig. 3). The analysis to compare the size of crystals between all organs regardless of growth stages showed significant differences ($H = 293.6$; $n = 91$, $df = 6$, $P < 0.001$; Table 2a). In the calyx, crystal size was significantly different compared to other vegetative and reproductive organs ($P < 0.01$ with stem, $P < 0.001$ with all other organs; Table 2b). The organs with larger crystals (cotyledon and root) were not significantly different from each other ($P > 0.05$), but were different compared with the other organs ($P < 0.001$; Table 2b). Stems and leaves were not differentiated by the size of crystals of calcium oxalate, as well as leaf

Table 1. Size of calcium oxalate crystals (μm) in vegetative and reproductive organs of soybean at different growth stages.

growth stage	cotyledon	leaf primordia	leaf midrib	leaf blade	stem	root	flower calyx	pod
vegetative (S12)	l: 21.3 ± 3.5 w: 5	l:12.2 ± 3.4 w: 5	l: 13.8 ± 3.2 w: 5	l: 15.4 ± 5.2 w: 5	l: 18.1 ± 5.0 w: 7.3 ± 3.3	l: 22.8 ± 6.6 w: 2.5–5.0	–	–
reproductive (S61)	–	–	l: 12.6 ± 2.8 w: 5	l: 17.2 ± 3.8 w: 5	l: 14.2 ± 5.1 w: 5.7 ± 1.9	l: 19.7 ± 4.3 w: 8.4 ± 2.1	l: 17 ± 3.4 w: 7.5	–
maturity (S89)	–	–	a	a	l: 10.7 ± 1.6 w: 5.4 ± 1.0	l: 18.8 ± 3.5 w: 8.5 ± 1.6	–	l: 11.7 ± 1.9 w: 6.7 ± 1.5

l = length; w = width.
[a]Leaves at the mature stage were absent at the time of sampling.

Table 2. Kruskal–Wallis test (a) and non-parametric multiple comparison test (b) on the comparison of crystal size between organs, regardless of their growth stage.

Kruskal–Wallis test (a)

	cotyledon	leaf primordia	leaf	stem	root	flower calyx	pod	total
N	91	91	91	91	91	91	91	637
mean	21.3	12.2	14.5	14.6	21	17	11.7	16
var	11.1	15.7	20.8	23.8	31.8	14	2.9	30
median	20	12.5	15	15	20	15	12.5	15
rank sum	45,668.5	16,774.5	24,703	25,134.5	42,815.5	33,662.5	14,444.5	203,203
rank mean	501.9	184.3	271.5	276.2	470.5	369.9	158.7	319

Non-parametric multiple comparison test (b)

	cotyledon	leaf primordia	leaf	stem	root	flower calyx	pod
cotyledon		10.46***	−9.24***	−8.36***	−0.6n.s.	7.09***	11.65***
leaf primordia	6.94×10^{-7}		3.92**	3.66**	9.34***	−7.70***	0.2n.s.
leaf	6.94×10^{-7}	1.66×10^{-3}		−0.01n.s.	−7.66***	−4.36***	5.22***
stem	6.94×10^{-7}	4.66×10^{-3}	1		−7.09***	−3.91**	4.75***
root	0.99	6.94×10^{-7}	6.94×10^{-7}	6.94×10^{-7}		5.27***	10.47***
flower calyx	6.94×10^{-7}	6.94×10^{-7}	2.52×10^{-4}	1.77×10^{-3}	3.52×10^{-6}		9.77***
pod	6.94×10^{-7}	0.99	4.36×10^{-6}	4.09×10^{-5}	6.94×10^{-7}	6.94×10^{-7}	

χ^2: 293.6; df: 6.
***$P < 0.001$; **$P < 0.01$; n.s. $P > 0.05$.

primordia and pods ($P > 0.05$), but the size was significantly different compared to other organs ($P < 0.01$ and $P < 0.001$; Table 2b). The comparison of crystal size between different growth stages showed that the size of calcium oxalate crystals in leaves and roots did not vary between the vegetative, reproductive and mature stages ($P > 0.05$), while in stems the size of the crystals decreased significantly from vegetative stage to maturity ($P < 0.01$). In leaves of the reproductive stage, crystals distributed along the midrib differed significantly from crystals associated with other vascular bundles in leaf blades (W = 6977; $P < 0.001$; Table 1, Fig. 3); while in leaves of the vegetative stage, no diferences were observed (W = 604; $P > 0.05$).

Generally, calcium oxalate crystals were associated with vascular bundles in all the organs analysed (Fig. 3). In cotyledons, crystals were randomly distributed in the mesophyll (Fig. 3A–C). In leaf primordia and leaves, prismatic crystals were distributed along the midrib, and secondary and tertiary vascular bundles (Fig. 3D–H). In stems, crystals were located in the sclerenchyma strands closely associated with the phloem and in the phloem (Fig. 3I–M); while in roots, crystals were associated with the parenchyma of the cortex beside vascular tissue (Fig. 3N–P). No calcium oxalate crystals were observed in flower petals, while in the calyx, crystals were located in the mesophyll as well as along vascular bundles (Fig. 3Q–T). In pods, crystals were distributed along the midrib of the carpel and randomly in parenchyma of the mesocarp (Fig. 3U–Z).

Quantification of calcium oxalate crystals

The Kruskal–Wallis test showed that there were significant differences in crystal density among the different organs, without taking into account the growth stage or different zones of the leaves (mibrid and blade; H = 45.3, n = 15, df = 4, $P < 0.001$; Table 3a). The pods were the organs where more crystals per mm^2 were quantified, and they differed significantly ($\bar{x}$: 1112 crystals·mm^{-2}; $P < 0.001$; Table 3) from all the other vegetative and reproductive organs (Tables 3b and 4, Fig. 4). Cotyledons, the organs with the lower density of calcium oxalate crystals per mm^2, differed significantly from the other organs ($\bar{x}$: 153 crystals·mm^{-2}; $P < 0.001$), except for the leaves ($\bar{x}$: 340 crystals·mm^{-2}; $P > 0.05$; Tables 3b and 4, Fig. 4). There were significant differences in crystal density between leaves from vegetative (293.2 ± 353.5 crystals·mm^{-2}) and reproductive (409.1 ± 376.5 crystals·mm^{-2}; H: 5.18, $P < 0.05$; Table 4)

Table 3. Kruskal–Wallis test (a) and non-parametric multiple comparison test (b) on the comparison of crystal density between organs, regardless of their growth stage.

Kruskal–Wallis test (a)

	cotyledon	leaf primordia	leaf	flower calyx	pods	total
N	15	15	15	15	15	75
mean	153.1	468	339.8	350	1112.2	484.6
var	7051.9	58,586	58,062.9	12,609.3	190,698.1	171,977.9
median	137.8	377.6	316.3	346.9	867.3	362.2
rank sum	214	632.5	471	537	995.5	2850
rank mean	14.3	42.2	31.4	35.8	66.4	38

Non-parametric multiple comparison test (b)

	cotyledon	leaf primordia	leaf	flower - calyx	pod
cotyledon		−4.04***	2.09n.s.	−3.96***	−4.66***
leaf primordia	4.96×10^{-4}		−1.34n.s.	1.12n.s.	−3.92***
leaf	0.22	0.66		−0.45n.s.	−4.39***
flower alyx	7.04×10^{-4}	0.79	0.99		−4.66***
pod	3.01×10^{-5}	8.39×10^{-4}	1.07×10^{-4}	3.06×10^{-5}	

χ^2: 45.3; df: 4.
***$P < 0.001$; **$P < 0.01$; n.s. $P > 0.05$.

Table 4. Net contribution of calcium oxalate crystals to the soil at different growth stages of the life cycle of soybean.

organs at growth stages	no crystals·mm^{-2}	area of the organ (mm^2)	number of organs·plant^{-1}	plant biomass (kg·ha^{-1})	no plants·m^{-2}	contribution to soil: no crystals·ha^{-1}
cotyledons (Vegetative, S12)	151	88	2	173.3	52	138.2×10^4*
leaf primordia (Vegetative, S12)	468	94	3		52	686.3×10^4*
leaves (Vegetative, S12)	293	972	5		52	74×10^6
leaves (Reproductive, S61 and Mature, S89)	409	4320	46	8493.9	52	42.3×10^6*
pods (Mature, S89)	1112	410	32	8975	52	76.3×10^7*
Net contribution of calcium oxalate crystals during a life cycle of soybean (Sum of *):						81.4×10^7

All values represent averages.

stages. However, the same comparison with cotyledons showed significant differences only with leaves at the reproductive stage (Z: 2.7, $P < 0.05$).

In leaves, there was a different pattern of biomineralisation between the midrib and the blade (H: 45.57, $P < 0.001$). The midrib was the section of the leaf with higher calcium oxalate crystal density. The comparison between leaves of different stages of growth showed that there were no differences between the midrib of vegetative and reproductive leaves (654.4 ± 330.7 and 772.3 ± 311.4 crystals·mm^{-2}, respectively), but there was a significantly higher density of crystals in the blade of leaves at the reproductive stage compared with leaves at the vegetative stage (142.9 ± 65.9 and 67.4 ± 39.8 crystals·mm^{-2}, respectively; Z: −3.65, $P < 0.01$).

Estimation of calcium oxalate crystal production and input into the soil from soybean plants

The average number of plants estimated in the field was 52 plants·m^{-2}. The net contribution of calcium oxalate crystals to the soil was calculated from all the organs (vegetative and reproductive) except stems and roots, where the crystal production was very rare (Table 4). In the vegetative stage (cotyledons, leaf primordia and leaves), a contribution of 82.3×10^6 crystals·ha^{-1} was estimated from a total vegetal biomass of 173.3 kg·ha^{-1} (Table 4). In the reproductive stage (leaves), the net contribution was 42.3×10^6 crystals·ha^{-1}, calculated from a soybean biomass of 8493.9 kg·ha^{-1} (Table 4). In this phenological stage, the production of calcium oxalate crystals was underestimated since it was not possible to quantify the crystal density in the calyx because of its small size and irregular morphology. At the mature stage (leaves and pods), the vegetal biomass was 8975 kg·ha^{-1}, and the contribution of calcium oxalate crystals was 80.5×10^7 crystals·ha^{-1} (Table 4).

Since during harvesting only the seeds are extracted, the net contribution of calcium oxalate crystals from a life cycle of soybean was calculated from the mature stage, including the cotyledons and leaf primordia, which become part of the straw in the early stages (Table 4). In this case, the net contribution of crystals was 81.4×10^7 crystals·ha^{-1} (Table 4).

DISCUSSION

Although there are reports of the presence of calcium oxalate crystals in soybean, these have focused on seeds (Ilarslan *et al.* 1997, 2001) or leaves (Cervantes-Martinez *et al.* 2005), so this work represents the first report on the description and quantification of calcium oxalate crystal production in vegetative

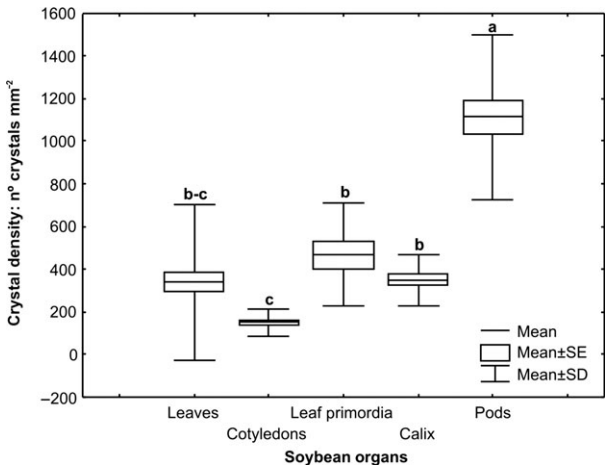

Fig. 4. Mean calcium oxalate crystal density (no crystals·mm^{-2}) in vegetative and reproductive organs of soybean. Different letters mean significant differences ($P < 0.001$).

and reproductive organs of soybean throughout the different growth stages of its life cycle.

Morphology and distribution of calcium oxalate crystals

According to Broadley *et al.* (2003), the mean relative shoot calcium (Ca) content in angiosperms ranges between −0.75% and 5.62% DW. The Fabales has intermediate levels of within-order variance in shoot Ca content, and the Fabaceae has intermediate values (−0.26% and 2.72% DW; Broadley *et al.* 2003). Generally, there are three physiotypes defined on Ca nutrition in plants: oxalate plants (precipitate Ca as oxalate salt), calciotrophes (contain high concentrations of free Ca^{2+}) and potassium plants (high K/Ca ratio). Although the Fabales is generally defined as a calciotroph, the presence of calcium oxalate crystals in Fabaceae is widely reported (Franceschi & Horner 1980; Zindler-Frank 1987; Jáuregui-Zúñiga *et al.* 2003; Duarte & Wolf 2005; Jáuregui-Zúñiga *et al.* 2005; Borrelli *et al.* 2009; He *et al.* 2012, 2013).

Although there are reports on the presence of different calcium oxalate crystal morphologies in Fabaceae (Erbano & Duarte 2012; He *et al.* 2012, 2013; Brown *et al.* 2013), twinned kinked prismatic crystals are the most common morphology associated with this family and to the Faboideae subfamily (Franceschi & Horner 1980; Zindler-Frank 1987; Ilarslan *et al.* 1997; Jáuregui-Zúñiga *et al.* 2003, 2005; Cervantes-Martinez *et al.* 2005; Duarte & Wolf 2005; Borrelli *et al.* 2009; He *et al.* 2012, 2013; Erbano & Duarte 2012; Brown *et al.* 2013), as was observed in this work on soybean plants.

In plants, calcium oxalate crystals occur in two hydration states: monohydrated ($CaC_2O_4 \cdot H_2O$, whewellite) and polyhydrated ($CaC_2O_4 \cdot (2 + X)H_2O$, weddellite; Franceschi & Horner 1980). Prismatic crystals found in soybean should be monohydrated (whewellite) as previously reported for *Phaseolus vulgaris* and soybean seeds (Ilarslan *et al.* 1997; Jáuregui-Zúñiga *et al.* 2003). These prismatic crystals belong to the monoclinic system, which is characterised by different monohydrated crystal morphologies (Verrecchia *et al.* 1993). In addition, weddellite (polyhydrated form) is the metastable form of calcium oxalate, so it is less widely distributed in plants than the more stable form, whewellite (Arnott 1995; Monje & Baran 2002).

We have observed the presence of calcium oxalate crystals in all vegetative and reproductive organs of soybean, even in developing organs such as cotyledons and leaf primordia. These biomineralisations might function as localised Ca sinks, preventing the accumulation of Ca in the apoplast of cells and allowing them to develop normally (Franceschi & Nakata 2005). Generally, all the prismatic crystals were associated with vascular bundles, particularly vascular bundle sheaths, as previously observed in leaves of soybean and *P. vulgaris* (Jáuregui-Zúñiga *et al.* 2003; Cervantes-Martinez *et al.* 2005). This tissue location could be explained as: (i) calcium distribution to the whole plant through the xylem (Prychid & Rudall 1999); (ii) calcium precipitation in cells surrounding the vascular bundles, preventing Ca accumulation around the chlorenchyma cells and ensuring their celular functions (Franceschi & Nakata 2005); and (iii) the dependence of calcium oxalate crystal morphology on the rate of calcium entry to cells; specifically, prismatic crystals develop when calcium transport through the cell is limited by lignification of their cell walls, as occurs in cells associated with vascular tissues (Borchert 1984). We observed that crystals in pods had the same morphology, but lower size compared with crystals in other vegetative and reproductive organs of soybean. Altamirano *et al.* (2014) reported the same pattern in several aquatic species. They showed that fruits have smaller crystals, but that crystal morphology changes in reproductive organs with respect to vegetative ones.

Comparing organs at different growth stages, we observed that in stems, although there are few crystals, their size falls with plant maturity. Probably this is related to the mobilisation of calcium for crystal production in reproductive organs (calyx, seed and pod), not only for nutritional and structural requirements, but also for the role of crystals as a herbivore defence (Molano-Flores 2001; Korth *et al.* 2006), especially taking into account the importance of these organs in the reproductive success of the species. In leaves, particularly at the reproductive stage, calcium oxalate crystals in the blade were longer than in the midrib, probably due to the structural function of these biomineralisations as they contribute to keeping the blade straight (Franceschi & Nakata 2005). In addition, the length: width ratio of crystals increased from primary veins to terminal ones, as previously reported in leaves of soybean (Cervantes-Martinez *et al.* 2005).

Quantification of calcium oxalate crystals and estimation of their production and input into the soil from soybean plants

The crystal density of soybean showed the same pattern reported in aquatic species from the southeast of the Pampean Plain (Altamirano *et al.* 2014). In these species, as we observed in soybean, the fruits have the larger crystal density and smaller crystal size compared with others vegetative and reproductive organs of the same species (Altamirano *et al.* 2014). The density of prismatic crystals has been reported in *Hydrocotyle bonariensis* (18,530 crystals·mm^{-2}) and *Rumex crispus* (7202 crystals·mm^{-2}; Altamirano *et al.* 2014), both values higher than the crystal density observed in pods of soybean (1112 crystals·mm^{-2}). The increase in calcium oxalate crystal density in fruits could be closely related to the biological functions of these organs, since the crystals could act as source of

calcium, ensuring normal growth and development of embryos because, aside from representing a method to store calcium, the oxalate is necessary for protein synthesis (Ilarslan *et al.* 1997, 2001). In addition, the importance of these biomineralisations in preventing damage from insects had been reported (Molano-Flores 2001; Korth *et al.* 2006), so the crystals might provide protection during the development and maturation of seeds. Cotyledons were the organs with the lowest crystal density, probably because most of the crystals were re-utilised by the embryos as besides representing a method for calcium storage, oxalate is neccesary for protein synthesis (Ilarslan *et al.* 2001).

Even though the crystal density in leaves of soybean is important, there have been reports of higher values (19.5 crystals·cm^{-2}) in *P. vulgaris* (Fabaceae-Faboideae; Jáuregui-Zúñiga *et al.* 2005) and *Sida rhombifolia* (Malvaceae): 6.45 crystals·cm^{-2} (Molano-Flores 2001), as well as lower densities (0.14–0.39 druses·cm^{-2}) in leaves of some aquatic species (Altamirano *et al.* 2014). Some reports on calcium oxalate crystal production in native and exotic arboreal species distributed in the southeast of the Pampean Plain showed that the crystal density was much higher compared to that of herbaceous species (Borrelli *et al.* 2009). There are reports of crystal density in phyllodes of *Acacia melanoxylon* (Fabaceae-Mimosoideae, 29,000 prismatic crystals·cm^{-2}), in leaves of *Celtis ehrenbergiana* (Celtidaceae, 24,000 druses·cm^{-2}) and *Eucalyptus globulus* (Myrtaceae, 12,000 druses·cm^{-2}; Borrelli *et al.* 2009). In leaves a larger proportion of the crystals was associated with the midrib compared to vascular bundles in blades. This is because calcium is distributed to the whole plant *via* the xylem, and its accumulation in cells of vascular tissue ensures the normal functioning of clorenchyma cells (Prychid & Rudall 1999; Franceschi & Nakata 2005).

Taking into account the importance of soybean and previous works on the decrease in organic matter, clay and structural stability in typic Argiudolls of the southeast of the Pampean Plain due to their agricultural and horticultural management, we consider that the contribution of calcium from soybean during its life cycle (81.4×10^7 crystals·ha^{-1}) is necessary to maintain the physico-chemical and biological properties of the soils. Calcium oxalate crystals are an important source of calcium to the soil solution, especially in soils where climatic conditions, mineralogy and degree of weathering cannot explain the calcium content (Pinilla *et al.* 1993; Borrelli *et al.* 2009). Hence, the crystals are necessary for the formation of stable organo-mineral complexes that influence soil aggregate stability (Porta Casanellas *et al.* 1999).

In sumary, *Glycine max* (soybean) produces prismatic calcium oxalate crystals in all vegetative and reproductive organs, resulting in a potencial input of about 81.4×10^7 crystals·ha^{-1} to the soil during its life cycle. Generally, the crystals are associated with vascular bundles. In cotyledons and the calyx, they are also located in the mesophyll, while in pods they are also distributed within parenchyma of the mesocarp. Pods are the organs with higher calcium oxalate crystal density, but of smaller size. However, cotyledons are the organs that produce the larger crystals, but in lesser amounts. This work represents the first report on the characterisation, production and potential input of calcium oxalate crystals from soybean plants to soils. The study therefore has biological and botanical, as well as biogeochemical and pedological relevance. These results also contribute to knowledge of the amount of calcium oxalate crystals involved in the process of Ca recycling through cultivated vegetation in fields from humid plains at medium latitudes.

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