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Calcium oxalate crystals of some *Crataegus* (Rosaceae) species growing in Aegean region

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Abstract: In this study, Ca oxalate crystals were isolated from the leaves and X-ray diffraction identified them as weddelite in *Crataegus pontica* C. Koch, *C. stevenii* Polar., *C. monogyna* ssp. *monogyna* Jacq. in *C. orientalis* var. *orientalis* Pallas ex Bieb. both whewellite and weddelite crystals were found. Although there were some differences among the soluble and insoluble oxalate contents, they were not notable in the species of *C. stevenii* (10%; 18%), *C. orientalis* (12.4%; 15%), *C. monogyna* (12.9%; 13%), whereas in *C. pontica* the difference was so great with the lowest soluble (4%), and highest (28%) insoluble oxalate content. Crystals have tetragonal or prismatic shape in general but tetrahedral kinked and straight shapes were seen in *C. orientalis*, tetragonal aggregates in *C. stevenii*, and also pseudo-tetrahedral cordate (heart) shape were found in *C. monogyna* ssp. *monogyna* and *C. pontica*. As the crystal biomineralization is under genetic control, this characteristic hydration state of crystals of *Crataegus orientalis* var. *orientalis* must be important for systematic phylogenetics.

Key words: *Crataegus*; calcium oxalate; crystals

Introduction

Calcium oxalate is a common biomineral in plants, occurring as crystals of various shapes either in monohydrate (whewellite) form or di-(or tri-) hydrate (weddelite) form (Arnott 1981). Calcium oxalate crystals are widespread in flowering plants, including both dicotyledons and monocotyledons. They have been identified in the leaves and other tissues in more than 215 families of the flowering angiosperms (Mc Nair 1932; Franceschi & Horner 1980; Ward et al. 1997). Morphology and distribution of the crystals vary widely among the genera and often between closely related species. The three main types of calcium oxalate crystals occur in monocotyledons: raphids, styloids and druses, although intermediates are sometimes recorded. The presence or absence of the different crystal types may represent useful taxonomic characters. For instance, styloids are characteristic of some families of Asparagales, particularly the Iridaceae, where raphids are entirely absent. The presence of styloids is therefore a synapomorphy for some families (e.g. Iridaceae) or groups of families (e.g. Philydraceae, Pontederiaceae and Haemodoraceae) (Prychid & Rudall 1999). There are only 11 published reports of crystals in the large family of Rosaceae. Except for an undocumented note on raphids in *Quillaja*, only druses and prismatics are known. The presence of raphids has been used to separate the subfamilies of Rubiaceae (Lersten 1974) and subgenera of *Prunus* (Lersten & Horner 2000). Also the patterns of crystal distribution and morphology are compared among the taxa and are evaluated with respect to phylogeny and biogeography in the genus *Gosypium* (Shockey et al. 2001).

Among the biological functions of the crystals regulation of bulk-free calcium levels in tissues and organs e.g. in the leaves of *Sida* (Moleno-Flores 2001), and a constitutive defense mechanism in secondary phloem of conifers (Hudgins et al. 2003) take place. A function in physical protection against grazing animals is implicated by the size, shape and location of crystals in *Claoxylon sandwicense* leaf. A large calcium oxalate crystal idioblast has grown by intrusive growth between palisade and spongy mesophyll cells and spans the entire cross section of the leaf. The idioblast has a single large styloid crystal with sharply pointed ends (Franceschi 2001). The needles of calcium oxalate can even have grooves and barbs, which are thought to be responsible for channelling toxins or anchoring the needles in the wound (Sakai et al. 1972; Sakai et al. 1984; Kuballa et al. 1981; Schmidt & Moult 1983; Doaigey 1991).

This study was carried out to characterize the types, shapes, and concentration of calcium oxalate crystals isolated from the leaves of four different species of *Crataegus* growing in Aegean Region in Turkey.

Material and methods

Four *Crataegus* species were collected in the Aegean Region in Turkey. *C. monogyna* ssp. *monogyna* Jacq. were collected in the National Park of Dilek Islands in Kuşadası, *C. stevenii* Pojark. in the Spil Mountain in Manisa, *C. orientalis* var.

orientalis Pallas ex Bieb. in the Balıkesir-Susuz Mountain and *C. pontica* C. Koch in the Uşak-Eşme Mountain in May. The fruity specimens were collected in the same sites in July to make the correct determination of species because of the apomixis problem between the *Crataegus* species. They were immediately rinsed into 70% ethyl alcohol.

Leaf clearing method

Leaf specimens were taken out from the 70% ethanol and left in 95% ethanol for one night. To dissolve and remove non-wall organic materials around the crystals the leaves were treated with 2.5% Clorox [(sodium hypochloride (NaOCl)] for 1–2 days. After these treatments the cleared leaves were washed with distilled water and dehydrated through a graded ethanol series (70% and 95% each for 15 min) then ethanol:xylene (1:1 v/v) for 15 min, pure xylene (for 10 min). Thin cross-sections were taken from these leaves for examining in polarizing microscopy. Whole leaves were mounted in Permount on slides, coverslipped, and examined under SEM.

Crystals in the leaf cross-section clearings were viewed with Leitz Wetzlar Orthoplan microscope with planachromatic lenses, and fitted with polarizing filters. Kodak Ektachrome 64 T and Techpan films were used to record the images.

Determination of oxalate concentration

The leaves of four *Crataegus* species were collected and their fresh masses recorded after drying at 60 ℃ for 1–2 days. Some of them were analyzed for total oxalate (soluble and insoluble) estimation. The remaining leaves were cleared (see previous section) and dried at 60 ℃. These samples were analyzed for insoluble oxalate (Ca oxalate). After drying, both the cleared and non-cleared samples were individually blended in mL of distilled water. Two milliliters of sample diluent EDTA (ethylenediamine-tetraacetic acid) were than added to the 2 mL of blended sample and mixed, and the pH was adjusted to values between 5 and 7. Oxalate concentration in each sample was determined using SIGMA Urinalysis Diagnostics Kit (Procedure Number 591, SIGMA) protocol: oxalate reagents were warmed to 37 ℃; tubes were labeled for blank, control, standard and sample; 1 mL oxalate reagent A [DMBA(3-dimethylamino benzoic acid + MBTH (3-methyl-2-benzothiazolinone hydrazone), pH 3.1] was added to each tube; 50 μL of sample were added to each sample tube; 50 μL deionized water were added to the blank and control tubes; 50 μL of oxalate standard were added to the standard tube, and 0.1 mL of oxalate reagent B (oxalate oxidase and peroxidase) was added to all tubes and immediately mixed by gentle inversion. All tubes were incubated at 37 ℃ for 5 min., the absorbance (A = the concentration of oxalate in sample is determined by comparing absorbance of sample with that of oxalate standard) of blank, control, standard, and sample were determined at 590 nm in a Perkin-Elmer Lambda UV/VIS spectrophotometer. Measurements were taken two to three times to check repetitiveness of the instrument. Corrected absorbances (A) were determined by substracting blank absorbance from absorbance reading of standard, control and the sample. Calculations to determine oxalate concentration in milligrams and percentage dry mass were determined using the SIGMA Urinalysis Diagnostics Kit. Data obtained in total, insoluble and soluble oxalate contents were analyzed by applying the analysis of variance Anova Tukey (Stell & Torrie 1980).

Isolation of crystals, SEM and powder X-ray diffraction methods

Whole leaves were processed according to Horner & Zindler-Frank (1982), and enzyme solution containing 5% cellulase and 1% pectolyase was used to aid in the release of crystals from the surrounding cell and crystal walls. A JEOL JSM-5200 scanning electron microscope (SEM) operated at 20 kV and 200°A was used to visualise isolated crystals in the leaves. Specimens were sputter-coated with gold and secondary electrons recorded. Images were recorded on Kodak TMAX 120 film. Whole leaves with isolated crystals were attached to a glass slide with vaseline and X-ray diffraction analysis was done with Jeol JSDX-100 S4 X-ray diffractometer apparatus using Cu X-ray tubes and Ni filter in 32 kV, 22 mA. The diffraction pattern was compared with ASTM (American Society for Testing and Materials) data for the hydration forms of Ca oxalate.

Results

All the *Crataegus* species investigated showed significant numbers of Ca oxalate crystals in their leaf tissues. The crystal shapes were tetragonal and prismatic in general, and also tetragonal crystals were kinked and straight in *C. orientalis* (Fig. 1b). The crystals often appear as pairs and crystal aggregates in clearings (Fig. 1a in *C. stevenii*, and in *C. orientalis*) (typically in two adjacent cells). The Z axis of each crystal is usually oriented perpendicular to the long axis of leaves (Fig. 1c). X-ray diffraction analysis of the crystals indicated that Ca oxalate dihydrate (weddelite) crystals were present

Table 1. Diffraction data of *Crataegus* species with corresponding ASTM data.

		ASTM data		Our results	
		d Å	I/I_1	d Å	I/I_1
C. pontica (14–769) $(COO)_2Ca \cdot H_2O$ Ca oxalate hydrate		3.60	60	3.50	58
C. orientalis	(14–769) Ca oxalate hydrate	5.45	100	5.45	100
	(20–231) $CaC_2O_4 \cdot H_2O$ Whewellite	5.93	100	5.90	100
C. stevenii (14–769)		4.95	40	4.90	47
C. monogyna (14–769)		3.60	60	3.50	84

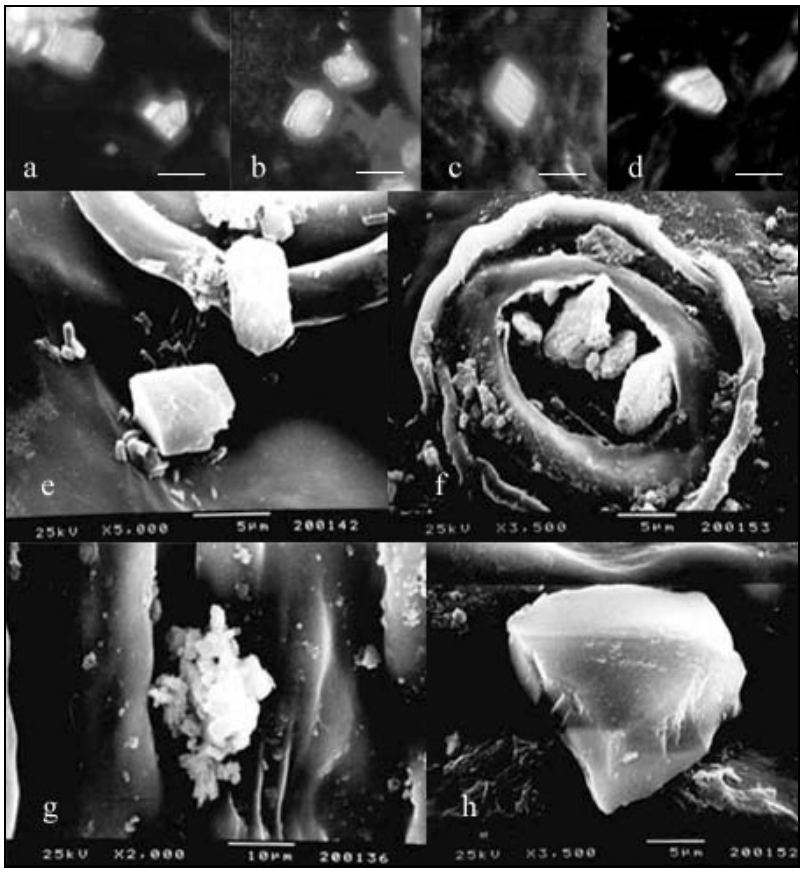

Fig. 1. Crystal types in *Crataegus* species visualized with polarizing microscopy. a – crystal pairings and aggregates in *C. stevenii*; b – kinked twins in *C. orientalis*; c – straight tetragonal in *C. orientalis*; d – prismatic in *C orientalis*. Bar = 66 μm. Various crystal shapes in SEM. e – tetragonal in *C. orientalis*; f – tetragonal crystals in the stoma cell of *C. monogyna*; g – tetragonal crystal aggregates in *C. pontica*; h – pseudo-tetrahedral cordat (heart shaped) crystal in *C. monogyna*.

in all of the species investigated while Ca oxalate monohydrate (whewellite) peak results were seen only in *C. orientalis* leaves together with weddelite (Table 1). The shapes were prismatic, pseudotetrahedral or cordate (heart shaped) in *C. pontica* and *C. monogyna* ssp. *monogyna* (Figs 1g,h). Ca oxalate crystals were observed to discharge from the stomata of *C. orientalis* and *C. monogyna* ssp. *monogyna* plants like the kidney stones in human (Figs 1e,f).

Total oxalate (soluble and insoluble) concentration (in percentage) per dry mass (milligrams) of leaves and insoluble oxalate (i.e., Ca oxalate) concentration (in percentage) per cleared leaves were calculated (Fig. 2) using the SIGMA Urinalysis Diagnostics Kit. The difference between total oxalate and insoluble oxalate represents soluble oxalate (Fig. 2). The analysis showed that total oxalate concentration reached 32% of the dry mass of the uncleared leaves of *C. pontica* with the highest value of which three quarters were insoluble Ca oxalate. *C. stevenii* (28%), *C. orientalis* (27%) and *C. monogyna* ssp. *monogyna* (26%) followed this total value consequently. The measurements of insoluble Ca oxalate support the visual data on the presence and size of crystals in cleared leaves representing a significant build up and change in Ca oxalate/oxalate concentration between the *Crataegus* species (Table 2).

Standard deviation was calculated from the measurements of oxalate as mmol/L in leaves of different species.

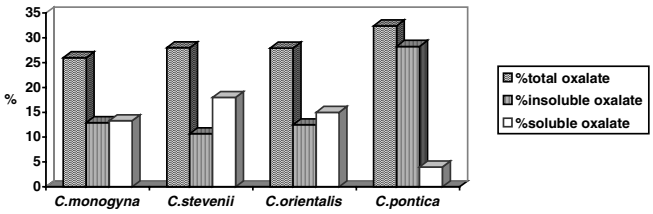

Fig. 2. Percentage of total, insoluble, and soluble oxalate per milligram of dried and cleared leaves of *Crataegus* species (*C. monogyna*, *C. stevenii*, *C. orientalis*, *C. pontica*). Total oxalate was determined in dried leaves and the insoluble (Ca oxalate) was determined in the cleared leaves. The difference represents soluble oxalate.

Discussion

Given the myriad possibilities, individual plant species typically display quite specific anatomical, morphological, and developmental patterns of crystal accumulation, reflecting genetic regulation of crystal formation. Crystal morphology and distribution are usually similar within specific taxa and differ among divergent taxa to the extent that provide key characters for systematics. Cultured plant tissues typically produce crystals

Table 2. Oxalate contents of four *Crataegus* species in mmol L^{-1} (mean ± SD).

Mean and Standard Deviation (mmol/L)	*C. monogyna*	*C. stevenii*	*C. orientalis*	*C. pontica*
Total oxalate	0.2621 ± 0.007^{bcd}	0.2881 ± 0.003^{ad}	0.2795 ± 0.0005^{ad}	0.3227 ± 0.006^{abc}
Insoluble oxalate	0.1292 ± 0.002^{bd}	0.1068 ± 0.0001^{acd}	0.1249 ± 0.004^{bd}	0.2829 ± 0.0001^{abc}
Soluble oxalate	0.1329 ± 0.002^{bcd}	0.1816 ± 0.002^{acd}	0.1554 ± 0.006^{abd}	0.0371 ± 0.004^{abc}

a – for *C. monogyna*, b – for *C. stevenii*, c – for *C. orientalis* and d – is statistically important for *C. pontica*.

morphologically identical to those characteristic of the intact plant (Kausch & Horner 1982).

Several studies have analyzed the hydration state of the crystals from a number of different plants, which include ribbon plant (Pennisi et al. 2001), cactus (Monje & Baran 2002) and water lattuce (Volk et al. 2002). Among them in cacti, species of different subfamilies have different hydration state of crystals, e.g. *Opuntioideae* have whewellite crystals whereas *Cereoideae* have weddelite. On the contrary, two hydrate forms of crystals (monohydrate and dihydrate) existed in *Dracaena sanderiana* as raphide crystals (Pennisi et al. 2001). As the latter example represents the monocotyledons, it will be interesting to find the two hydration forms in *Crataegus orientalis* as a member of dicotyledons. Also *C. orientalis* with druses and prismatics showed synapomorphy being a member of Rosaceae (subfamily of Maloideae) with two hydration forms in one species. In *Draceanae* (Liliales) only twin raphids were seen while only druses, twin and straight tetragonal prismatics were observed in *C. orientalis* (Rosales). Some dicots are known to be more similar to monocots than they are to other dicots. The dicots are paraphyletic (that is they contain some, but not all, descendants of the most recent common ancestor of that group), whereas the monocots can be defined by several synapomorphies (Anonymous 2005). But kinked twins were firstly observed in soybean as whewellites in Leguminosae (Ilarslan et al. 1997). Kinked twins were distinctive characteristics of *C. orientalis* var. *orientalis*. Although twinning was proposed to enhance crystal stability and strength (Arnott & Webb 2000), its biological significance seems here genetically controlled (Nakata & Mc Conn 2000). In legumes the control of the morphology of a crystal is a genetically regulated process and a simple point mutation can drastically alter the size and shape of the crystal (Nakata 2002). So, the occurrence of two crystal hydration forms in *C. orientalis* seems to be important for systematic phylogenetics. One might speculate that *C. orientalis* and the other *Crataegus* species ancestors investigated could have followed different pathways at an early stage in evolution.

In *C. pontica* the highest total Ca oxalate and the lowest soluble oxalate values were observed (Franceschi & Loewus 1995). Even though these crystals are more abundant in *Crataegus* species, this difference may be the consequence of the differential availability of calcium in the soil, since there is a positive correlation between calcium supply and the amount of crystals of calcium oxalate in plant tissues (Zindler-Frank 1995). This supported the consideration that calcium oxalate was formed only to maintain low soluble levels of the potentially toxic oxalic acid.

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