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ARTICLE

Calcium oxalate crystals in *Conyza canadensis* (L.) Cronq. and *Conyza bonariensis* (L.) Cronq. (Asteraceae: Astereae)

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ABSTRACT Calcium oxalate crystals (CaOx) are found in most organs and tissues of many plant species. In this study the morphology and distribution of CaOx crystals in *Conyza canadensis* (L.) Cronq. and *Conyza bonariensis* (L.) Cronq. belonging to Asteraceae were investigated. CaOx crystals display a similar distribution in organs and tissues of *C. canadensis* and *C. bonariensis.* For the identification of CaOx crystals a histochemical technique (using silver nitrate and rubeanic acid) was applied to the cleared organs and tissues. Crystals in cleared organs and tissues were viewed using an Olympus photomicroscope fitted with polarizing filters. The sample tissues were also investigated with a scanning electron microscope. CaOx crystals were found in stems, leaves, petals, ovaries, and styles of two species, but no crystals were observed in filament or other tissues. Druses were observed in the stem epidermis and cortex cells and leaf epidermis cells and mesophyll layers in both species. They were also determined in corolla and style cells. The pith parenchyma cells of stem had needle-shaped and bipyramidal crystals. Styloid crystals were present in the ovary of both species. Raphides were not observed in both taxa. This study provides additional information about the presence of CaOx crystals in Asteraceae.
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KEY WORDS

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Conyza bonariensis

The genus *Conyza* Less. belonging to tribe Astereae includes herbs, shrubs and trees and comprises over 60 species which are mainly distributed in tropical and subtropical areas. The Astereae tribe is the second largest tribe of Asteraceae, with over 170 genera and 3000 species worldwide (Bremer 1994). Most widespread two species of *Conyza* genus in Europe and Turkey are *Conyza canadensis* (L.) Cronq. and *Conyza bonariensis* (L.) Cronq. (Grierson 1975; Tutin et al. 1976). *Conyza* spp. contain alkaloids, saponins, tannins, glycosides, phenols, flavonoids, oil and sphingolipids (Kong et al. 2001; Mukhtar et al. 2002). Today no literature is found about the existence of calcium oxalate crystals in *Conyza* species.

Calcium oxalate (CaOx) crystals are formed from environmentally derived calcium and biologically synthesized oxalate and they are common for most tissues and organs of plants (Franceschi and Horner 1980; Prychid and Rudall 1999; Nakata 2003). The shape and location of these crystals within a taxon are often very specific and they are used in classification of plants (Franceschi and Horner 1980; Lersten and Horner 2000). CaOx crystals occur in different plant tissues including leaves (Horner and Zindler-Frank 1982; Lersten and Horner 2000), stems (Franceschi and Horner 1980), roots (Horner et al. 2000), and seeds (Webb and Arnott 1982; Ilarslan et al. 1997, 2001). CaOx crystals also occur in floral organs including ovaries (Dormer 1961; Tilton and Horner 1980), anthers (Horner 1977; Horner and Wagner 1980; Meric and Dane 2004) and petals (Meric and Dane 2004). The only place where crystals have not been seen is the pollen (Tilton and Horner 1980). CaOx crystals are widely distributed in plant kingdom and found in over 215 families (Franceschi and Horner 1980; Molano-Flores 2001). However, their functional significance remains unclear, although various functions have been attributed to them. CaOx crystals give protection against foraging animals (Molano-Flores 2001), bind toxic oxalate (Borchert 1984) and are involved in in-plant Ca regulation (Franceschi 1989), salt stress and homeostasis (Hurkman and Tanaka 1996), light gathering (Franceschi and Horner 1980) and detoxification of heavy metals (Nakata 2003).

Conyza Less. genus belongs to Asteraceae (about 1500-1600 genera and 23000 species) which is one of the greatest families of plant kingdom (Bremer 1994). Crystals in Asteraceae were shown by a few previous studies (Dormer 1961; Horner 1977; Meric and Dane 2004). The most comprehensive review of crystal types and distribution for a single family [Zindler-Frank 1987 (Leguminosae); Wu and Kuo-Huang 1997 (Moraceae)] lacks substantial documentation. The present study is a part of an ongoing project aiming to bring to light CaOx crystals in Asteraceae and this study aims to determine the CaOx crystals in *C. canadensis* and *C. bonariensis*. Thus additional research is needed to better determine CaOx crystals in other taxa belonging to Asteraceae.

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Table 1. Morphologies and locations within the tissues of calcium oxalate crystals in *Conyza canadensis* and *Conyza bonariensis.*

Location	*Conyza canadensis*	*Conyza bonariensis*
stem – epidermis	druse (3.61 ± 0.33 µm)	druse (3.55 ± 0.33 µm)
stem – cortex	druse (1.89 ± 0.17 µm)	druse (2.20 ± 0.22 µm)
stem – pith parenchyma	needle-shaped, bipyramid (6.15 ± 1.94 µm, 4.44 ± 1.16 µm)	needle-shaped, bipyramid (7.79 ± 3.02 µm, 4.58 ± 1.20 µm)
leaf – epidermis	druse (4.36 ± 0.46 µm)	druse 4.03 ± 0.48 µm
leaf – mesophyll	druse (2.24 ± 0.20 µm)	druse (3.07 ± 0.29 µm)
corolla	druse (3.16 ± 0.43 µm)	druse (4.92 ± 0.83 µm)
anther	---	---
filament	---	---
ovary	styloid (6.48 ± 1.83 µm)	styloid (6.93 ± 1.33 µm)
style	druse (3.45 ± 0.53 µm)	druse (4.76 ± 0.62 µm)

Materials and Methods

Vegetative and generative organs of *Conyza canadensis* (L.) Cronq. and *Conyza bonariensis* (L.) Cronq. were used as materials. Plants were grown in the Botanical Garden of Biology Department (Trakya University).

For light microscopy, materials were fixed in the mixture of ethyl alcohol and glacial acetic acid (3:1 v/v) at room temperature overnight and then the mixture was changed to 70% ethyl alcohol. The hand-sections of fixed stems and leaves were carried out. Corollas, anthers, filaments, ovaries and styles were dissected out of florets under a stereo microscope. The samples were treated with 2.5% Clorox (sodium hypochlorite) for 4 h. After graded ethyl alcohol series, the samples were infiltrated with xylene, mounted in entellan on slides, and covered with cover slips (Ilarslan et al. 1997). Crystals were observed under bright-field optics with or without polarizers on an Olympus Photomicroscope and images were captured with an Olympus digital camera. The histochemical determination of CaOx crystals was carried out according to the procedure of Yasue (1969) on the tissue clearings. Cleared samples were immersed in 5% aqueous $AgNO_3$ for 15 min, then thoroughly rinsed in distilled water. The samples were stained with saturated rubeanic acid (dithio oxamide) (Fluka, Germany) in 70% ethanol for 1 min. All examinations were made on ten plants at the anthesis stage.

For scanning electron microscopy (SEM), samples fixed in the mixture of ethyl alcohol and glacial acetic acid were dehydrated in a graded ethyl alcohol series to absolute ethyl alcohol and dried at the critical point (Bio-Rad E 3000, Hertfordshire, UK). Dried specimens were coated with gold (Bio-Rad SC 502, Hertfordshire, UK), and examined using a Jeol JSM SEM (Jeol, Tokyo, Japan). Control samples were treated with 5% acetic acid, 10% hydrochloric acid, 3% nitric acid and 4% sulfuric acid (Molano-Flores 2001). All these tests confirmed that the crystals were calcium oxalate.

Measurements of crystals were performed using Image-Pro Plus, version 5.1 (Media Cybernetics, Silver Spring, MD). For analysis, the diameters of druses, the lengths of styloids and needle-shaped crystals, and the side lengths of bipyramidal crystals were measured. A hundred of crystals for each tissue and each crystal type were measured from randomly chosen 10 regions. Averages and standard deviations of data were calculated.

Results

Calcium oxalate crystals displayed a similar distribution in the tissues and organs of *Conyza canadensis* and *Conyza bonariensis.* Table 1 shows the distributions and types of CaOx crystals in both species.

The clearing technique removed all the cytoplasm except for cell walls and CaOx crystals and the crystals were observed easily under light microscope with bright and polarized light. Druses were present in the stem epidermis cells of *C. canadensis* and *C. bonariensis* (Fig. 1A). Almost each stem epidermis cell had a single druse crystal. The diameters of these crystals were almost similar in both species (3.61 ± 0.33 µm for *C. canadensis* and 3.55 ± 0.33 µm *C. bonariensis*). Druses were also observed in parenchyma cells of the stem cortex (Fig. 1B). The diameters of these crystals were smaller than those of stem epidermis cells (1.89 ± 0.17 µm for *C.*

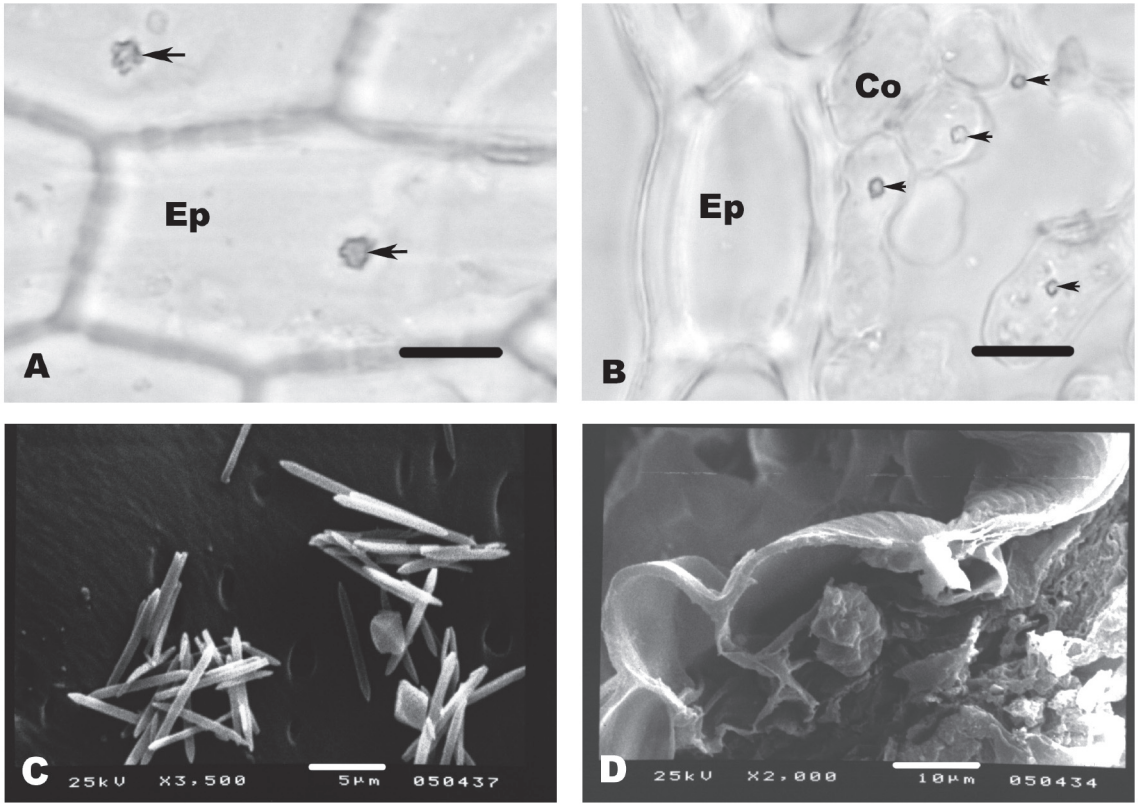

Figure 1. Calcium oxalate crystals in the tissues of *Conyza canadensis*. (A) Druse crystals in the stem epidermis cells of *C. canadensis* (arrows). (B) Druses in the stem cortex cells of *C. canadensis* (arrows) (in cleared tissues with bright light). (C) SEM photograph of needle-shaped and bipyramidal crystals in the pith parenchyma cells of stem of *C. canadensis*. (D) SEM photograph of druse crystal in the leaf epidermis cell of *C. canadensis.* Scale bar= 10 µm (A, B, D), scale bar= 5 µm (C). Ep, epidermis; Co, cortex.

canadensis and 2.20 ± 0.22 µm for *C. bonariensis*). The stem pith parenchyma cells had bipyramidal and needle-shaped crystals (Fig. 1C). The lengths of needle-shaped crystals were measured as 6.15 ± 1.94 µm for *C. canadensis* and 7.79 ± 3.02 µm for *C. bonariensis.* The side lengths of bipyramidal crystals were determined as 4.44 ± 1.16 µm and 4.58 ± 1.20 µm for *C. canadensis* and *C. bonariensis* respectively.

In leaves, druse crystals were observed in both epidermis and mesophyll cells of *C. canadensis* and *C. bonariensis*. Druses were present in both adaxial and abaxial epidermis cells of the leaves (Fig. 1D). The diameters of these crystals were measured as 4.36 ± 0.46 µm for *C. canadensis* and 4.03 ± 0.48 µm for *C. bonariensis*. They were also observed in the leaf mesophyll layers (Fig. 2A). The diameters of the druses within these cells were determined as 2.24 ± 0.20 µm and 3.07 ± 0.29 µm for *C. canadensis* and *C. bonariensis* respectively. Each leaf epidermis and mesophyll cell contained a single druse crystal.

A single druse crystal was determined in almost each cell of corollas in both species (Fig. 2B). The diameters of these crystals were measured as 3.16 ± 0.43 µm for *C. canadensis* and 4.92 ± 0.83 µm for *C. bonariensis.* Druses in corollas of *C. bonariensis* were bigger than of *C. canadensis.* Styloids were observed in each cell of ovaries in both species (Fig. 2C). The lengths of them were determined as 6.48 ± 1.83 µm and 6.93 ± 1.33 µm for *C. canadensis* and *C. bonariensis* respectively. The cells had single or a cluster of styloid crystals formed of a few of them. Druses were also observed in the style cells of the species (Fig. 2D). The diameters of these crystals were measured as 3.45 ± 0.53 µm for *C. canadensis* and 4.76 ± 0.62 µm for *C. bonariensis*. No crystals were found in the filament or other tissues. Localizations of CaOx crystals were also demonstrated by the Yasue (1969) procedure (Fig. 2B). The crystals stained brownish-black with this technique.

Discussion

CaOx crystals are present in almost every part of both vegetative and reproductive organs in plants and they are found in over 215 plant families (Franceschi and Horner 1980; Prychid and Rudall 1999; Molano-Flores 2001). Unfortunately, there are only a few studies related to the existence of them in Asteraceae (Dormer 1961; Horner 1977; Meric and Dane

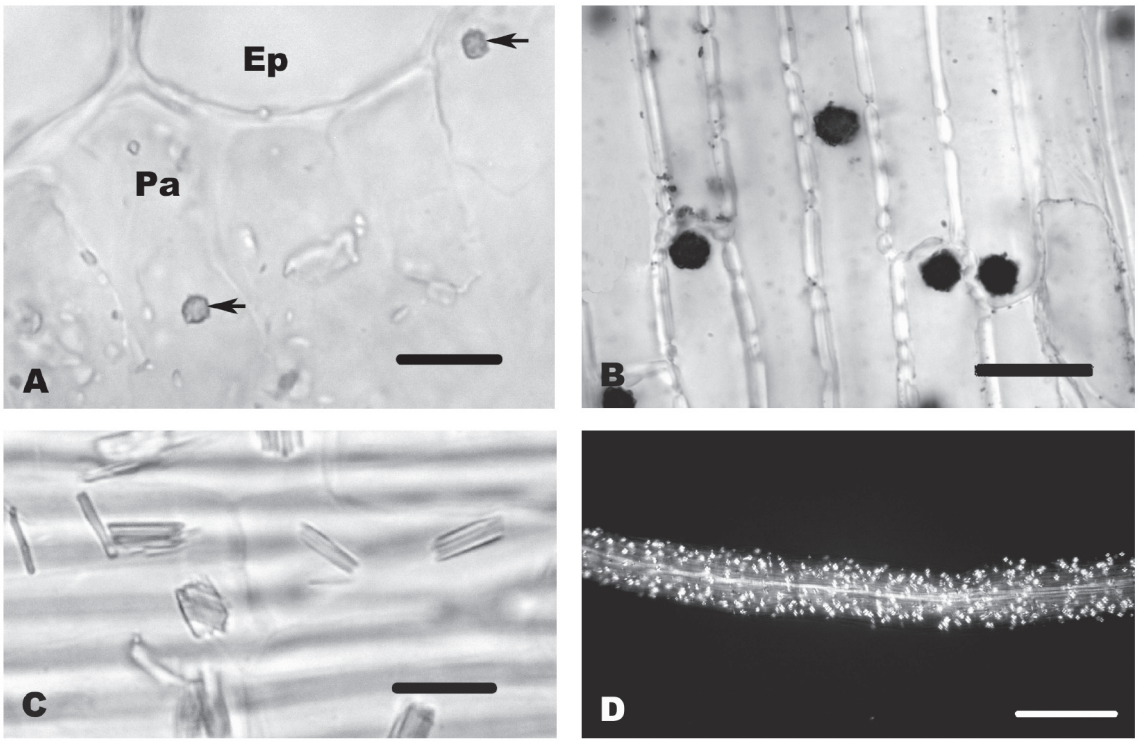

Figure 2. Calcium oxalate crystals in the tissues of *Conyza bonariensis*. (A) Druses in the leaf palisade cells of *C. bonariensis* (arrows) (in cleared tissues with bright light). (B) Druses in the corolla cells of *C. bonariensis* (stained with Yassue procedure). (C) Styloids in the ovary cells of *C. bonariensis*. (D) Druses in the style cells of *C. bonariensis* (with polarized light). Scale bar= 10 μm (A, B, C), scale bar= 100 μm (D). Ep, epidermis; Pa, parenchyma.

2004). In the present study, CaOx crystals were observed in *Conyza canadensis* and *Conyza bonariensis*. Druses were common in the tissues and organs of both taxa. They occurred in corollas, styles, leaves (epidermis and mesophyll) and stems (epidermis and cortex) of the two species investigated. The needle-shaped and bipyramidal crystals were present in the pith parenchyma cells of stems in both species. Styloids were only present in the ovaries of the specimens. No raphides were observed in the investigated tissues. Prychid and Rudall (1999) reported that druses are common in dicotyledons while raphides are widely found throughout monocotyledons.

Druses were observed in stem epidermis and cortex cells of *C. canadensis* and *C. bonariensis*. Franceschi and Horner (1980) reported that various crystals were present in the wood and parenchyma cells of some species belonging to Boraginaceae, Burseraceae, Combretaceae, Geraniaceae, Haloragaceae, Lauraceae, Leguminosae, Myrtaceae, Pinaceae, Polygonaceae, Rosaceae and Verbenaceae. Trockenbrodt (1995) determined CaOx crystals in the bark of *Quercus robur* (Fagaceae), *Ulmus glabra* (Ulmaceae), *Populus tremula* Salicaceae) and *Betula pendula* (Betulaceae).

The presence of CaOx crystals in leaves is common. In previous studies, crystals were determined in the epidermal cells of *Gleditsia triacanthos* (Leguminosae) (Borchert 1984) and *Stylosanthes guianensis* (Leguminosae) leaflets (Brubaker and Horner 1989). In addition Wu and Kuo-Huang (1997) observed druses in the epidermis of *Artocarpus altilis* (Moraceae) leaves and needles in the epidermis of *Ficus virgata* (Moraceae) leaves. Druses were determined in the mesophyll layers of *Actocarpus altilis, Cudrania cochinchinensis, Ficus virgata* and *Morus australis* (Moraceae) by Wu and Kuo-Huang (1997). Lersten and Horner (2000) reported that druses were the most common crystals in most *Prunus* species and calcium oxalate crystals in the leaves of this genus can be useful for the solution of the still unsettled systematics problems of *Prunus.* Kuo-Huang et al. (2007) suggested that druses crystals in the palisade cells of *Peperomia glabella* were utilized in the photosynthetic process and the diameters of these druses were related with light intensity.

Druse crystals were also found in corolla and style cells. Formation of this type of crystals was found to be related with changes in calcium levels within the plant (Nakata 2003). When calcium levels increased, druse crystal number and size also rapidly increased. When calcium levels decreased, number and size of druse crystals also decreased (Nakata 2003). Probably calcium is released from crystals for utilization in plant. Ca is an important component for cell wall synthesis and maintenance in higher plants and is essential

for the formation of the middle lamella. It is also related with the activation and/or stabilization of certain enzymes (Franceshi and Horner 1980). Serious abnormalities result from Ca deficiency in plants. The lack of Ca causes damage in membrane and organelle, changes in the cell wall and mitotic abnormalities (Franceshi and Horner 1980).

The presence of CaOx crystals in gynoecia was displayed in Dilleniaceae, Liliaceae, Palmae, Malvaceae, Cunoniaceae and Euphorbiaceae (Tilton and Horner 1980). Raphides are the most common type in gynoecia. But other crystal forms occur as crystal sand in carpels of *Bakeridesea* (Malvaceae) and also druses appear in several Cunoniaceae (Tilton and Horner 1980). In the ovaries of Asteraceae, CaOx crystals have been investigated in detail for the first time by Dormer (1961). Dormer (1961) determined prismatic crystals in the ovaries of *Centaurea scabiosa* (about 12 µm in length), *Silybum marianum* (about 45 µm in length), *Onopordon acanthium* (about 17x12 µm in sizes), *Carthamus tinctorius* and *Cirsium vulgare* (about 40 µm in length in both species). Also Dormer (1961) observed isodiametric crystals in the ovaries of *Arctium* sp. It appears that ovaries of Asteraceae contain crystals varying enormously in size. The investigated *Conyza* species include relatively smaller crystals (6.48 and 6.93 µm) than of previous studies (Dormer 1961). The present study aimed to determine CaOx crystals in *Conyza canadensis* and *C. bonariensis* belonging to the Asteraceae. It is probable that other members of Asteraceae might also contain crystals. Thus, further studies are necessary on the CaOx crystals in Asteraceae in order to determine the general value of crystals as a diagnostic feature for anatomical descriptions.

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