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Calcium Oxalate Crystals in Pistils and Stamens of Some Angiosperm Taxa

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Abstract
Calcium oxalate crystals that are present in almost all parts of the angiosperms have different morphological forms: as druses, prism, styloids, raphides and crystal sand. In the present study, occurrence, types and distribution of calcium oxalate crystals in the pistil and stamen of four angiosperm taxa including *Sternbergia lutea* (L.) Ker-Gawl. Ex Sprengel, *Bellevalia edirnensis* N. Özhatay & Mathew, *Trachelium jacquinii* (sieber) Boiss. subsp. *dalgiciorum* N. Özhatay & Dane and *Cyclamen coum* Miller. were investigated with light microscopy using paraffin and handmade sections. During the study, special attention was given to reveal the presence and absence of crystals in the stigma, style, ovary, anther and filament at different developmental stages of the pistil and stamen. To observe the crystals at different stages of another development, buds at different phases of microsporogenesis, and microgametogenesis were used. Two types of calcium oxalate crystals were identified in the examined plants. While raphide crystals were observed in *S. lutea* and *B. edirnensis*, druse crystals were observed in *Cylamen coum* and *T. jacquinii* subsp *dalgıciorium*. At the end of the study, it was concluded that presence and distribution of calcium oxalate crystals in the pistil and stamen depends on the developmental stages of the male and female organs.
Keywords: Druse crystals; Raphide crystals, *Sternbergia lutea, Bellevalia edirnensis, Trachelium jacquinii*. subsp. *dalgiciorum*

Bazı Angiosperm Taksonlarının Pistil ve Stamenlerinde Kalsiyum Oksalat Kristalleri

Özet
Angiosperm ve gymnospermlerin çoğu kısımlarında bulunan kalsiyum oksalat kristalleri, druz prizmatik, styloid, rafit ve kum kristalleri olmak üzere farklı morfolojik yapılara sahiptirler. Bu çalışmada, *Sternbergia lutea* (L.) Ker-Gawl. Ex Sprengel, *Bellevalia edirnensis* N. Özhatay & Mathew, *Trachelium jacquinii* (sieber) Boiss. subsp. *dalgiciorum* N. Özhatay & Dane and *Cyclamen coum* Miller taksalarına ait, pistil ve stamenlerde Ca-oksalat kristallerinin varlığı ve dağılımı ışık mikroskobunda parafin ve el kesitleri kullanılarak araştırıldı. Çalışma sırasında Ca-oksalat kristallerinin stigma, stilus, ovaryum, anter ve filamentteki varlıkları ve dağılımları, pistil ve stamenlerin farklı gelişim aşamaları gözönüne alınarak incelendi. Anter gelişiminin değişik aşamalarındaki kristalleri inceleyebilmek için, mikrosporogenezin ve mikrogametogenezin farklı aşamalarında olan tomurcuklar kullanıldı. Mevcut çalışmada, çalışılan bitkilerde rafid ve drus kristalleri olmak üzere sadece iki çeşit Ca-oksalat kristali görüldü. *S. lutea* ve *B. edirnensis* bitkilerinde rafit kristalleri gözlenmesine karşın, *Cylamen* coum and *T. jacquinii* subsp *dalgıciorium* bitkilerinde druz kriştalleri gözlendi. Çalışma sonucunda Ca-oksalat kristallerinin stamen ve pistildeki varlıklarının ve dağılımlarının dişi ve erken organın gelişim aşamalarına göre farklılık gösterdiği anlaşılmıştır.
Anahtar kelimeler: Druz kristalleri, rafit kristalleri, *Sternbergia lutea*, *Bellevalia edirnensis*, *Trachelium jacquinii*. subsp. *dalgiciorum*, *Cyclamen coum*

INTRODUCTION

Ca –oxalate crystals are naturally found in more than 215 higher plant families [1-3], including gymnosperms and angiosperms. In angiosperms, crystal formation generally takes place inside the vacuoles of specialized cells called idioblast, and it is usually associated with membranes, chambers, or inclusions found within the vacuoles. Crystal idioblasts can be distinguished from the non-crystal- forming cells of the same tissue by their different shapes, sizes and intracellular structures [4]. Different from angiosperms, in gymnosperms most of the crystals form in the cell wall [5]. Although the shape, size, location and number of crystals show variations among taxa, they have been classified into five main groups based on their morphology: as prism, druses, styloids, raphides and crystal sand [6,7]. According to many authors [8-10] differences in the shape, location, size, and other properties of plant crystals may be caused by the various physical, chemical and biological parameters such as light, temperature, pH, ion concentration and herbivory. However, many authors have stated that crystal formation within the cell is under genetic control [11]. Thus the shape and location of the crystals within a taxon are often very specific and may be represented as a taxonomic character [12-14].

Calcium oxalate crystals may be present in almost all parts of the plant. In higher plants, ca –oxalate crystals are located generally in the fundamental tissue of vegetative organs such as epidermis, mesophyll, phloem, [7, 22, 23], stems, seeds [11, 24-27], floral organs [28-30], anthers [31-34] and root nodules [35].

Although there are numerous studies on the presence of calcium oxalate crystals in the fundamental tissues of vegetative organs, there are only a few records about the occurrence of Ca –oxalate crystals in the generative organs. Therefore, in this study, the presence of ca-oxalate crystals in the pistil (ovary wall, style, and stigma) and the stamen of generative organs have been investigated.

The primary purpose of this study was (1) to document the existence and distribution of calcium oxalate crystals in the pistils and stamens of the selected plant species by correlating them with the successive developmental stages of the male and female organs and to support the hypothesis which suggests relationship between the presence of crystals and plant growth; (2) to provide data for taxonomic studies by revealing the location, types and distribution of calcium oxalate crystals in the generative organs of the studied plants.

MATERIALS AND METHODS

In this study, four plant species belonging to different families were collected from their natural habitats in Edirne Province (European Turkey). Collected species were identified by Feruzan Dane. The voucher specimens were kept in the herbarium of Trakya University (EDTU). Detailed information about the names and studied parts of the four taxa is given in table 1.

The flower buds and flowers which were obtained from examined plants were preserved in 70 % ethyl alcohol after being fixed in Carnoy's fluid (3:1 v/v, ethyl alcohol: acetic acid). For light microscopy study, clearing method, aceto-orcein squash method, and paraffin methods were applied.

Clearing method

In clearing method, cross sections were obtained by hand from the fixed samples and sections were treated with a solution of 5 % NaOH for 48 hours and were preserved in chloral hydrate for 2 hours. Chemical structure of crystals was identified by using 5 % $AgNO_3$ and 5 N HCl [36].

Squash method

In squash method, while first group of materials were treated in 45 % acetic acid for 10 minutes after being hydrolysed with 1N HCl for 5 minutes, second group of materials was waited in acetic acid of 45 % without being hydrolysed. Then both groups of materials were crushed in aceto-orcein.

Paraffin method

Flower buds at different developmental stages were embedded in paraffin and the hematoxylin and eosin staining method was applied to sections which were taken from the paraffin embedded buds by using microtome.

All the sections were examined and photographed with an Olympus Photo microscope.

RESULTS

In the present study, all of the examined plants were found to contain calcium oxalate crystals. While raphide crystals were observed in *S. lutea* and *B. edirnensis*, druse crystals were observed in *C. coum* and *T. jacquinii subsp dalgıciorium*. Types and distribution of crystals in each of the studied plant have been summarized in Table 2 and also have been described in details under separate subheadings.

Table 1. List of examined species and their collection data

Species	EDTU number	Collector	Date of collection	Location
Sternbergia lutea (L.) Ker-Gawl. Ex Sprengel (Amaryllidaceae)	7378	G. Dalgıç	16 June 1995	İzmir, Bozdağ
Bellevalia edirnensis N. Özhatay & Mathew Hyacinthaceae	7392	F. Dane	01 January 1998	Kırklareli, Pehlivanköy
Trachelium jacquinii (sieber) Boiss. subsp. *dalgiciorum* N. Özhatay&Dane (Campanulaceae)	5434	G. Dalgıç	04 June 1993	Edirne, Keşan, Mecidiye
Cyclamen coum Miller. (Primulaceae)	3104	G. Dalgıç	19 March 1989	Kırklareli, Vize

Table 2. Examined taxa, their crystal type and crystal's location

Taxa	Location	Crystal Type
Sternbergia lutea (L.) Ker-Gawl. Ex Sprengel	Pistil (style and ovary wall)	Raphide
Bellevalia edirnensis N. Özhatay & Mathew	Pistil, (style and ovary wall) Stamen (filament, anther endothecium cells)	Raphide
Trachelium jacquinii (sieber) Boiss. subsp. *dalgiciorum* N. Özhatay&Dane	Pistil (stigma and style)	Druse
Cyclamen coum Miller.	Pistil (ovary wall)	Druse

Sternbergia lutea **(L.) Ker-Gawl. Ex Sprengel**

To investigate the presence of Ca-oxalate crystals in the pistil and stamen of *S. lutea* during the all stages of development, various sized flower buds, at the different development stages were used. Occurrence of calcium oxalate crystals in the styles and stigma was investigated by using both squash and clearing methods. After the application of squash method, cells including raphide crystals were observed in the style but not in stigma, where only a few pollen grains were observed. To determine the specific locations of raphide crystals in the style, it was waited in clearing fluid and it was observed that long chains of raphide crystals were extending from the stigma to the ovary around the trachea. (Figure1). When the paraffin sections taken from the ovary were examined, it was seen that, there were raphide crystals in the cells underlying the epidermis of the ovary wall, between parenchyma cells and around the vascular tissue (Figure 2). When the ovaries, which were cleaned by using clearing method, were stained with $AgNO_3$ crystals, they became black (Figure 3).

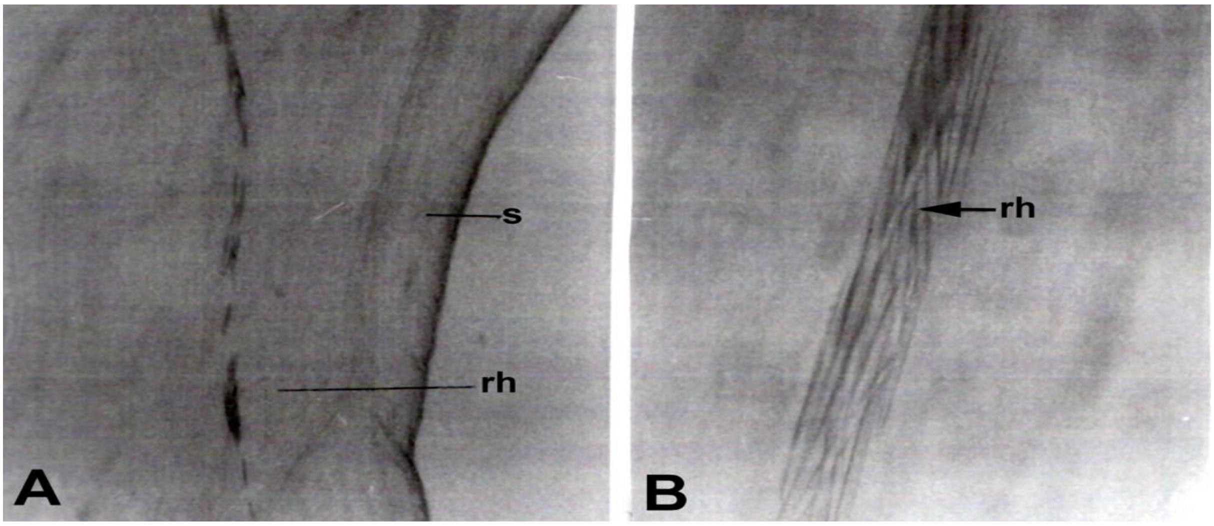

Figure 1. (A, B) Raphide crystal idioblast in the style of the *S. lutea* . A. Raphide crystal idioblast cells arranging in long chains.x 10; B. Idioblasts which include raphide crystals (s, style; rh, raphide crystals) (Cleaning method) x40.

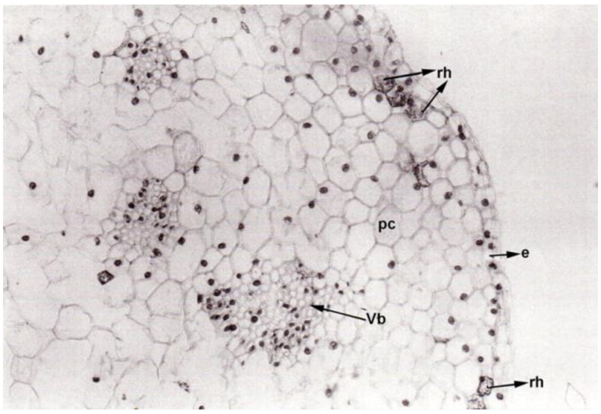

Figure 2. Crystal idioblasts in the cross section of the ovary of the *S. lutea*. (e, epidermis ; rh, raphide crystal idioblasts cells; vb, vascular bundle; pc, parenchyma cells) x 10.

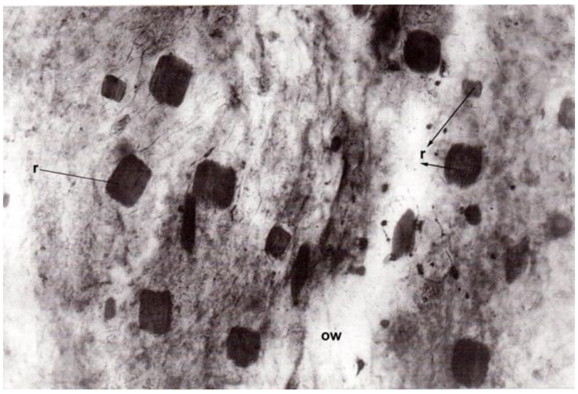

Figure 3. Different sized raphide crystal bundles in the ovary wall of the *S. lutea* which is stained by $AgNO_3$ (r, raphide crystal bundles; ow, ovary wall) x 20.

Bellevalia edirnensis **N. Özhatay & Mathew**

In *B. edirnensis*, occurrence of crystals in the pistil and stamen were investigated at the different stages of microsporogenesis and microgametogenesis. For this purpose, paraffin sections which were taken from the young and mature flower buds, and flowers by using microtome were examined. Raphide crystals were also observed in samples which were prepared by using squashed method.

Although the raphide crystals were observed in the style, stigma and the ovary of the pistil (Figure 4, 5), they were not present during the all stages of microsporogenesis. For example, crystals were not observed in the pistil until the dyad phase of microsporogenesis. In the tetrad stage, a few raphide crystal idioblasts were identified in the ovary wall and the style but not in the ovule. Raphide crystals were also observed at the microgametogenesis stage of pollen development. During pollen mitosis, the number of raphide crystal idioblast found in the ovary wall and style increased.

Moreover, specific location of crystals also showed variation during the different stages of pistil development. At the early stage of the pistil development, raphide crystal idioblasts were observed between meristematic cells, whereas, in the young pistil they were observed between parenchyma cells. Crystals were not observed in the ovule in any stage of development. In the stamens of *B. edirnensis*, raphide crystals were observed in the parenchyma cells of the filament and endothecium cells of the anther (Fig. 6). However, as in pistil, they were not present during the all stages of the development. Raphide crystals were identified in the stamen during the one nucleate microspore stage and later developmental stages. It was also determined that crystals which were found in the pistil and stamen could be stained with $AgNO_3$ (Figure 4-6).

Cyclamen coum **Miller**

In *C. coum*, occurrence of the calcium oxalate crystals were investigated only in the pistil, and druse crystals were observed in the ovary wall of the mature embryo sac (Figure 7).

Trachelium jacquinii **(sieber) Boiss** ***T. jacquinii*** **subsp.** ***dalgıciorum*** **N. Özhatay &Dane**

In *T. jacquinii* subsp.*dalgıciorum*, druse crystals were seen on the stigma during the pollen germination (Figure 8). When the flower buds at the different stages of microsporogenesis were squashed in aceto- orcein after the acetic acid treatment, druse crystals were observed in the stigma and styles during the all stages of the development.

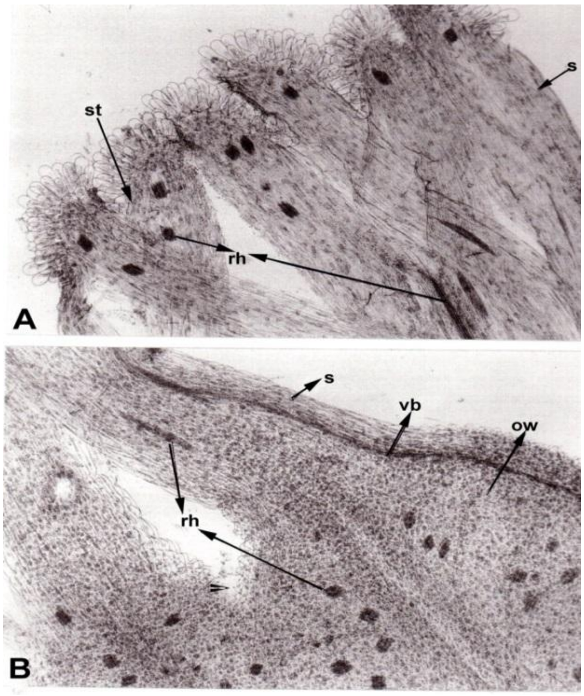

Figure 4. A- B. Raphide crystal idioblasts in the style and the ovary of the pistil of the *B. edirnensis* which was cleaned by the clearing method and stained with $AgNO_3$. (rh, raphide crystal idioblast cells; ow, ovary wall; s, style; st, stigma; vb, vascular bundle) A, B, X4

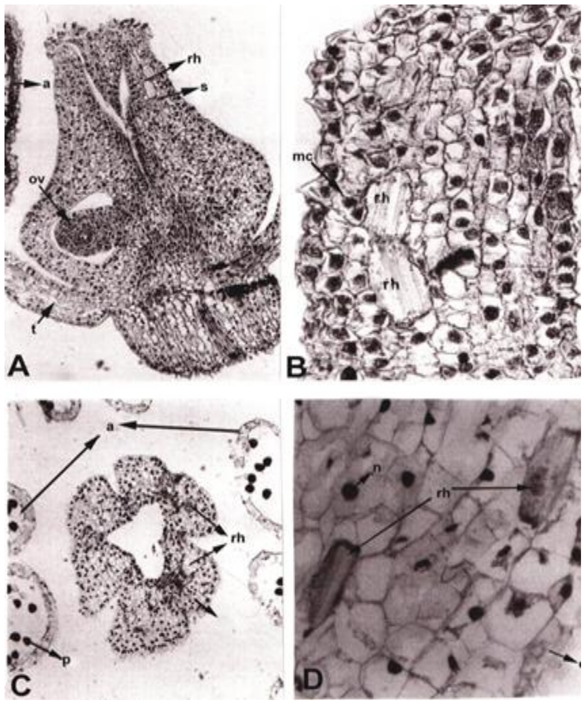

Figure 5. Cross section and longitudinal section of the young flower bud of *B. edirnensis* showing raphide crystal idioblasts in the style and the ovary. A) longitudinal section of the style; C) cross section of the style ; D) cross section of the ovary (rh, raphide crystal idioblast cells; ov, ovul; a, anther; t, tepal ; s, style; st, stigma; mc, meristematic cells; p, pollen; n, nucleus) A, B, x4 ; C, x10; D, x 40.

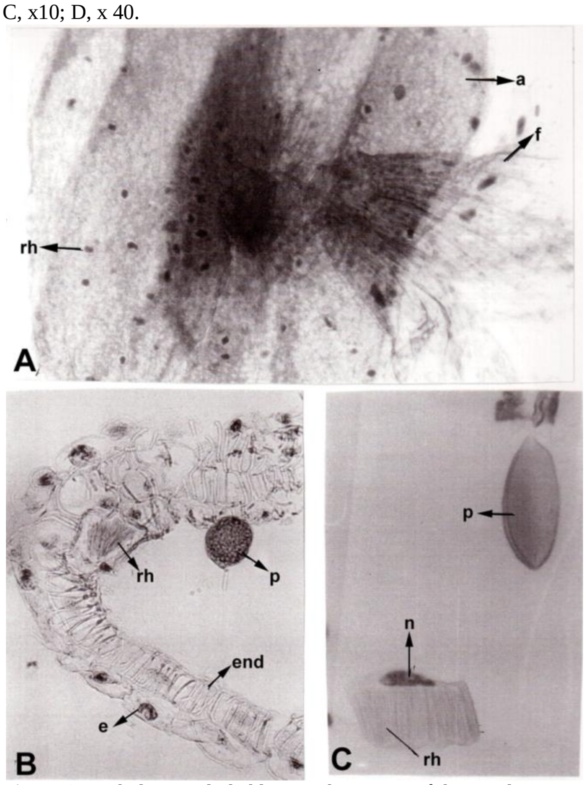

Figure 6. Raphide crystal idioblasts in the stamen of the *B. edirnensis*. A) Raphide crystal idioblasts in the anther and the filament of the stamen which was cleaned by clearing method and stained with $AgNO_3$; B) Cross section of the anther showing crystal idioblast cells between endothecium cells.(in paraffin section which was stained by hematoxylin - eosin staining method); C) pollen grain and raphide crystal idioblast cells (prepared by squash method and stained with aceto- orcein) A, x 4 ; B, x 20; C, x 40).

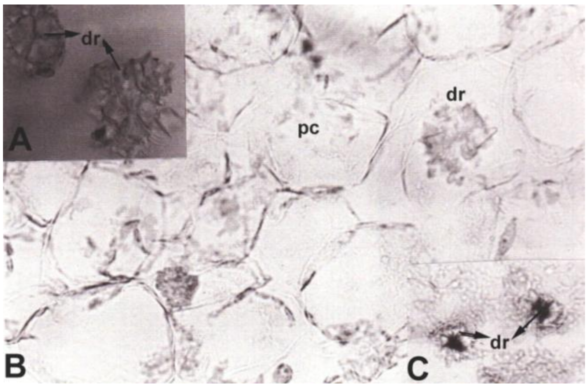

Figure 7. Druse crystal idioblasts in the ovary wall of the *Cyclamen coum* (dr, druse crystals; pc, parenchyma cells) A, B, x 40 ; C, x 20.

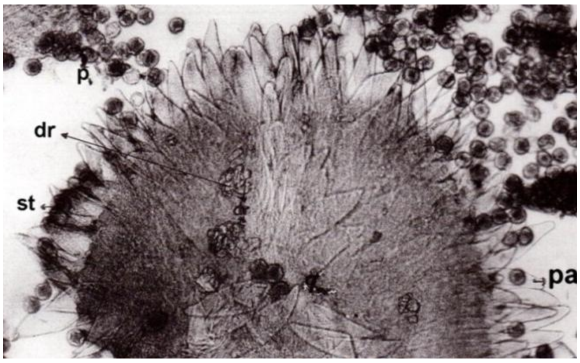

Figure 8. Druse crystals in the stigma of the *T. jacquinii* subsp.*dalgıciorum* (p, pollen; st, stigma; dr, druse crystals; pa, papilla cells) x 10.

CONCLUSION AND DISCUSSION

In the current study, crystal development in the generative organs of *B. edirnensis* , have been examined by correlating them with the different stages of anther development, and raphide crystals have been identified in its generative organs. Crystals which have been initially observed in the style and ovary wall at the tetrad phase of development have been also observed in the subsequent phases such as one-nucleate pollen, pollen mitosis, two-nucleate pollen and mature pollen grains. However, crystal formation has not been observed in ovules. Presence of raphide crystals in *B. edirnensis* is not a new finding for the family which it belongs, because occurrence of the raphide crystals in the members of the Hyacinthaceae has been known for a long time. But this type of crystals generally has been observed in vegetative organs [13], and there are only a few records about the presence of raphide crystals in generative organs [28-34]. For instance, presence of raphide crystals in the carpels of *Ornithogolum coudatum* has been shown by Tilton and Horner [28]. Different from the pistil, in the stamens of *B. edirnensis*, raphide crystals have been formed in one nucleate pollen stage and persist in later developmental stages. In preceding years, presence of crystals in anthers has been also reported in *Capsicum annuum* plant [31].

According to many authors crystals are found in anthers for three main purposes. These are: (1) as a product of metabolism, (2) to provide protection against predators, and (3) to provide the anther opening.

In the study of Horner and Wagner [31], observations on the degeneration of druse crystals in the stomium of *Capsicum annuum,* during the anther opening, has revealed that, they are effective in the anther opening. Horner and Wagner have proposed that [31] opening of an anther was caused by the presence of crystals in anther and pressure done by the oxaloacetic acid on endothecium cells.

Apart from the studies related with anther opening, studies on the possible protective role of raphide crystals in young leaves are also present [37].

In Amaryllidaceae existence of raphide crystals in generative organs have been reported in *Leucojum aestivum* [33] and *Galanthus* sp. [30]. Although occurrence of raphide crystals in root meristem cells of *S. lutea*, have been reported by Dane et al. [17], there is no report about the existence of raphide crystals in the generative organs of *S. lutea.* Therefore presence of raphide crystals in the stigma, style and ovary of *S. lutea* has been reported for the first time in this study. In the current study, occurrence of the raphide crystals in the style *of S. lutea,* in the young and mature developmental stages, has been proved. This finding is important, because reports on the occurrence of crystals in style are very scarce. To the best of our knowledge, the only record on the presence of crystals in the style is the study which was done to investigate the reason of the self-incompatibility of *Pontederia sagittata* [38]. However, in this study, purpose of the existence of crystals has not been stated. In *Pontederia sagittata*, raphide crystals idioblasts have been observed just beneath the epidermis. But in *S. lutea* and *B. edirnensis* raphide crystal idioblasts have been seen in parenchyma tissue. Detailed studies must be done in order to explain the functions of the crystals in the styles.

To understand the formation and functions of calcium oxalate crystals in plants, it is necessary to investigate their presence at different developmental stages. For this reason, various studies have been made in different plant organs which correlate the existence of crystals with plant growth. For example, in corms and root meristem cells, it has been shown that formation of the ca-oxcalate crystals is a dynamic process. In *Colasia esculant,* Sunel and Healy [39] have been observed that formation of the raphide crystals increases with increasing diameter of corms but after a certain diameter raphide crystal formation decreases. Additionally, In *Typha glacua*, Seago and Marsh [40] have observed that raphide crystal formation increased in the adventitious roots which was 5 mm length but as the roots became shorter formation of the raphide crystals decreased and aerenchyma formed**.** Moreover, degeneration of crystals has been observed in the stomium of the anthers of *Capsicum annuum* [31] and in the roots of the *Typha glauca* [40]. While degeneration of crystal idioblasts in *Typha glacua* has been related with the formation of the aerenchyma [40], in *Capsium annuum* it has been correlated with the opening of the stomium [31].

In addition to *B. edirnensis* and *S. lutea*, druse crystals have been also observed in the ovary wall of the mature embryo sac *C. coum* (Primulacea). Recently, oxalate crystals have been reported in the seeds of the some members of the Primulaceae [42].

During the morphological examination of pistil, druse crystals have been observed in the stigma *of T. jacquinii* subsp. *dalgıciorum* (Campanulaceae). Additionally, when the young and mature buds and flowers of *T. jacquinii* subsp. *dalgıciorum* examined by squashed method, druse crystals have been observed in the stigma and the styles.

To the best of our knowledge there is no record on the presence of the crystals in the members of the Campanulaceae. In this study, the presence of the Ca-oxalate crystals in the generative organs of *S. lutea*, *B. edirnensis*, *C. coum* and *T. jacquinii* subsp. *dalgıciorum* has been documented. Author thinks that further studies on calcium oxalate crystal formation in generative organs are necessary to establish the correlation between their existence and functions in different developmental stages and to contribute the understanding of their function.

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