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Calcium Oxalate Crystals in Some Species of the Tribe Inuleae (Asteraceae)

CILER MERIC*

Department of Biology, Trakya University, 22030, Edirne, Turkey

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In this study, calcium oxalate (CaOx) crystals were investigated and their morphology and distribution determined by light microscopy in tissues and organs of *Inula graveolens* (L.) Desf., *Pulicaria dysenterica* (L.) Bernh., *Filago eriocephala* Guss., *Logfia arvensis* (L.) Holup and *Logfia gallica* (L.) Coss. & Germ., which belong to tribe Inuleae (Asteraceae). CaOx crystals were identified in cleared organs and tissues by a histochemical technique using silver nitrate and rubeanic acid. Druses were observed in stem pith cells, leaf mesophyll cells and style cells of *I. graveolens*. In anther tissues, crystals were determined as styloids, and in the ovary they were identified as prismatics. No crystals were found in petal and filament cells of *I. graveolens*. Druse crystals were present in the filament and style cells of *P. dysenterica*; styloids were found in the endothecial tissues of anthers, and prismatic crystals in the ovary cells of this species. No crystals were found in petal, stem and leaf tissues of *P. dysenterica*. *F. eriocephala* and *L. arvensis*, and *L. gallica* had small prismatic crystals only in their ovaries. No crystals were observed in the other tissues of these species. This study represents additional data on the presence of CaOx crystals in Asteraceae.

Key words: Asteraceae, calcium oxalate crystals, Inuleae, *Inula, Pulicaria, Filago, Logfia*.

INTRODUCTION

Asteraceae is one of the largest families of the plant kingdom, with about 23,000 species (Bremer, 1994). It is spread worldwide, with numerous genera, and contains many crops and ornamental plants. The Inuleae tribe is comprised of shrubs, subshrubs and herbs (Anderberg, 1991). As a tribe it is relatively smaller than the others in the family, with about 500 species. Many of its species are antibacterial, antiseptic, anti-inflammatory, antiulcerogenic, antipyretic, diuretic, antidiabetic, antirheumatic, sedative, antispasmodic, antihaemorrhoidal or anthelmintic (Ali-Shtayeh et al., 1998; Alkofahi and Atta, 1999; Al-Dissi et al., 2001; Nickavar and Mojab, 2003). There are no literature reports on calcium oxalate crystals in species of the tribe Inuleae.

Calcium oxalate (CaOx) crystals occur quite commonly in the plant kingdom. They are found in over 215 plant families and distributed in organs such as stems, roots, leaves, floral structures and seeds (Franceschi and Horner, 1980; Tilton and Horner, 1980; Horner and Wagner, 1980, 1992; Prychid and Rudall, 1999; Horner et al., 2000; Lersten and Horner, 2006). The shapes of CaOx crystals vary and are commonly described as raphides, druses, styloids, prisms and crystal sand (Franceschi and Horner, 1980; Franceschi and Nakata, 2005). Crystals often form in the vacuoles of cells called crystal idioblasts, which are specialized for crystal formation (Foster, 1956). In some plants, crystals accumulate in the vacuoles of other cell types such as mesophyll, storage parenchyma and epidermal cells (Franceschi and Horner, 1980). In other cases they are deposited in cell walls, as seen in gymnosperms (Franceschi and Horner, 1980; Hudgins et al., 2003). Although their functional significance in plant development remains unclear, various functions have been attributed to them, including calcium regulation in plant cells (Franceschi, 1989; Kostman and Franceschi, 2000; Volk et al. 2002), protection against herbivory (Molano-Flores, 2001), detoxification of heavy metals or oxalic acid (Franceschi and Nakata, 2005), tissue strength, light gathering and reflection (Franceschi and Horner, 1980; Kuo-Huang et al., 2007).

The size, location and other properties of the crystals in plants may be affected by physical, chemical and biological conditions such as temperature, light, pressure, pH, ion concentration and herbivory (Franceschi and Horner, 1980; Molano-Flores,

*e-mail: cilermeric@trakya.edu.tr

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TABLE 1. Morphology (type and size in μm) and locations of calcium oxalate crystals within the tissues in Inuleae

Taxon	Stem (pith)	Leaf (mesophyll)	Corolla	Anther	Filament	Style	Ovary
Inula graveolens	druse diameter 10.00 ± 1.71	druse diameter 3.46 ± 0.37	–	styloid length 4.32 ± 0.69	–	druse diameter 3.66 ± 0.55	prismatic length 25.96 ± 4.64
Pulicaria dysenterica	–	–	–	styloid length 11.55 ± 2.75	druse diameter 3.92 ± 0.47	druse diameter 3.90 ± 0.60	prismatic length 24.67 ± 2.35
Filago eriocephala	–	–	–	–	–	–	prismatic length 12.06 ± 1.96
Logfia arvensis	–	–	–	–	–	–	prismatic length 14.32 ± 1.28
Logfia gallica	–	–	–	–	–	–	prismatic length 8.70 ± 1.14

2001; Kuo-Huang et al., 2007). However, it is thought that crystal formation within the cell is under genetic control (Ilarslan et al., 2001). Thus the type, presence or absence of crystals may be represented as a taxonomic character (Prychid and Rudall, 1999; Lersten and Horner, 2000). Unfortunately there are only a few studies related to their presence in Asteraceae (Dormer, 1961; Horner, 1977; Meric and Dane, 2004; Meric, 2008). The aim of this study is to provide additional information on the presence of CaOx crystals in Asteraceae.

MATERIALS AND METHODS

Plants were collected from natural habitats in Edirne Province (Turkey). For light microscopy, materials were fixed in ethyl alcohol and glacial acetic acid (3:1 v/v) at room temperature overnight and transferred to 70% ethyl alcohol. Hand-sections were made from fixed stems and leaves. Corollas, stamens, ovaries and styles were dissected from florets under a stereomicroscope. The samples were treated with half-strength Clorox commercial bleach (2.5% sodium hypochlorite) for 4 h. After washing in a graded ethanol series, the samples were infiltrated with xylene, mounted in entellan on slides, and covered with cover slips (Ilarslan et al. 2001). Crystals were examined in cleared tissues with a brightfield microscope (Olympus, Tokyo, Japan) and crossed polarizers. Selected images were captured with an Olympus (Tokyo, Japan) digital camera and processed in PhotoShop 7.0 (Adobe, San Jose, California).

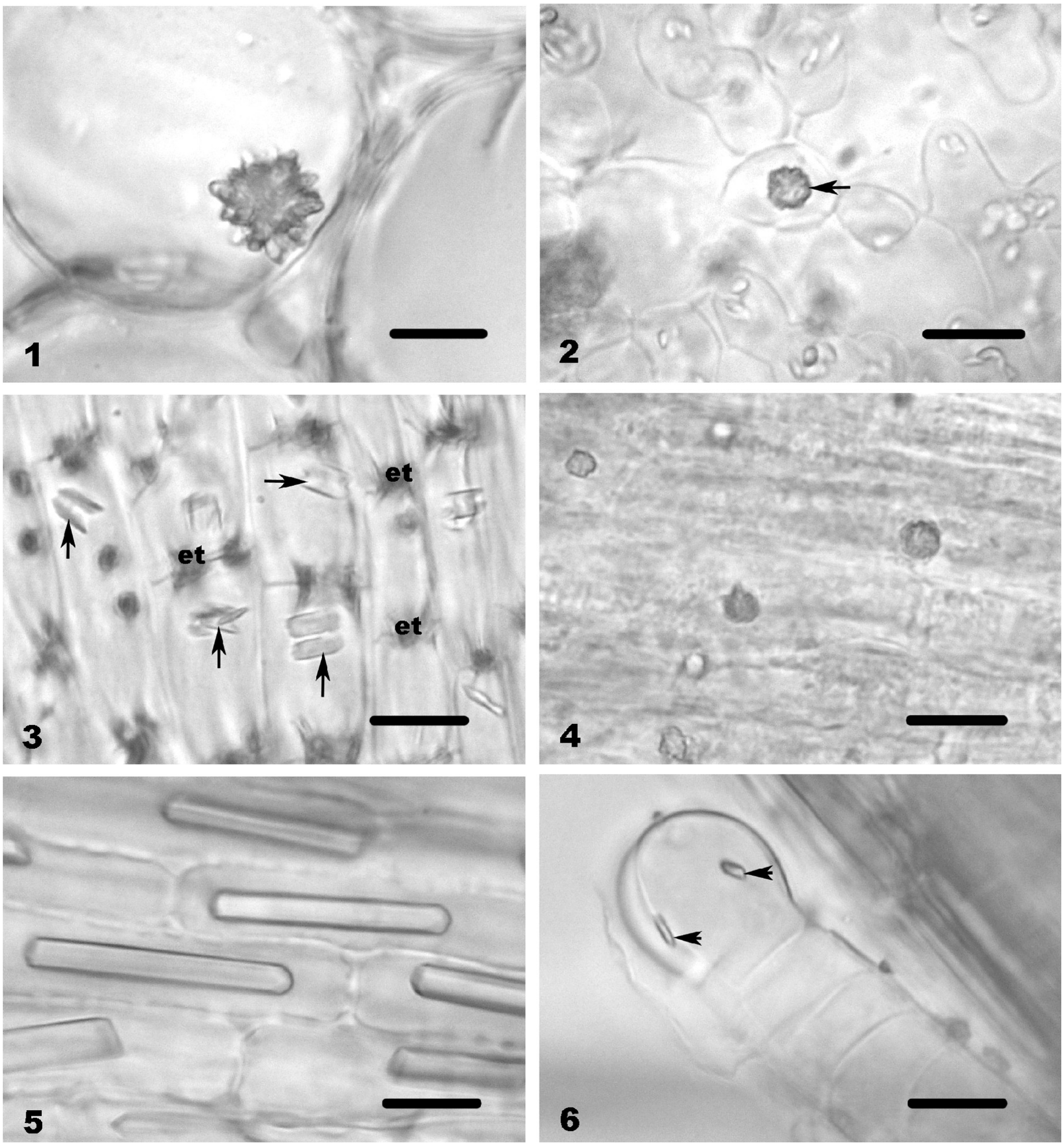
The crystals were measured using Image-Pro Plus, version 5.1 (Media Cybernetics, Silver Spring, MD). The analyses included druse diameter, styloid length and prismatic crystal length. One hundred crystals for each tissue and each crystal type were measured from 10 randomly selected regions. Averages and standard deviations of the raw data were calculated.

Histochemistry of calcium oxalate (CaOx) crystals was determined according to Yasue (1969) in the cleared tissues. Cleared samples were immersed in 5% aqueous $AgNO_3$ (Merck) for 15 min and thoroughly rinsed in distilled water. The samples were stained with saturated rubeanic acid (Dithiooxamide, Sigma) in 70% ethanol for 1 min. Control samples were treated with 5% acetic acid, 10% hydrochloric acid, 3% nitric acid and 4% sulfuric acid (Molano-Flores, 2001).

RESULTS

The calcium oxalate (CaOx) crystals in all cleaned tissues were easily observed since the clearing technique removed all the cytoplasm except for cell walls and crystals. They were examined by LM (brightfield and polarized light). Their morphology and distribution in tissues are shown in Table 1.

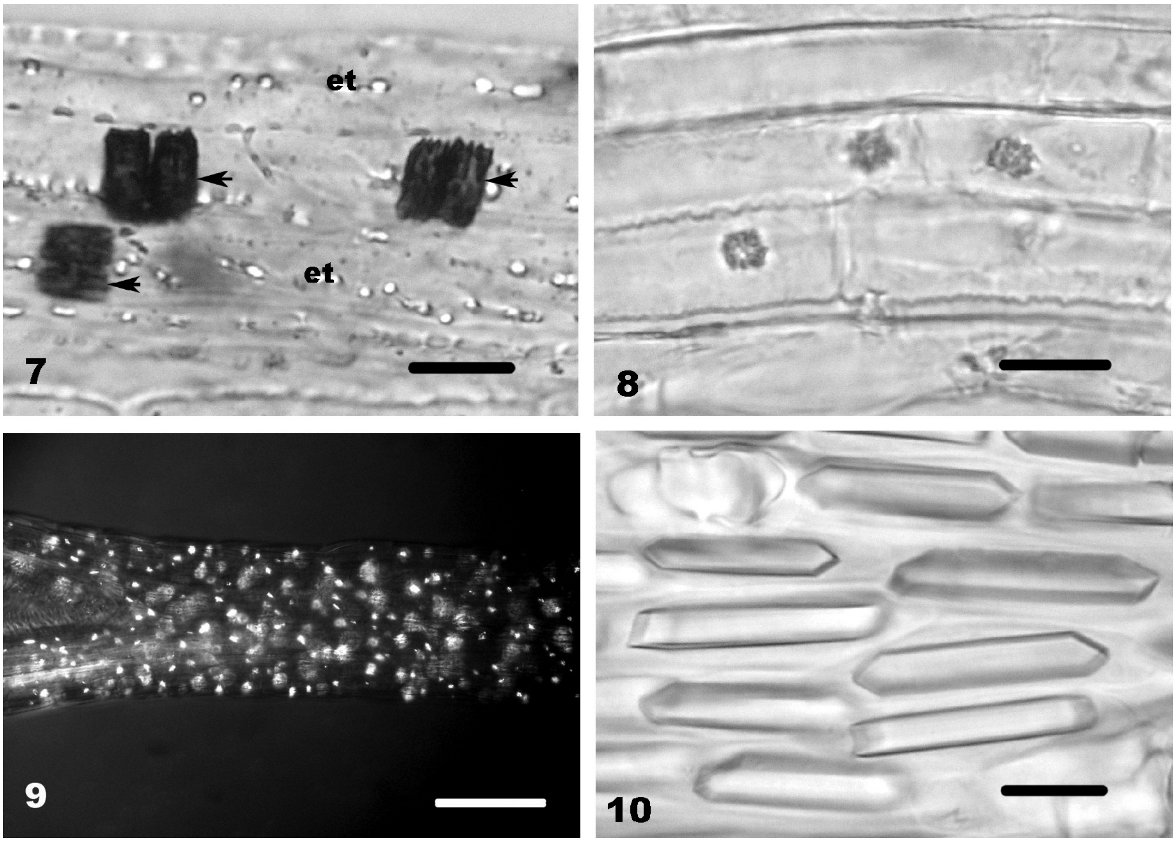
The distribution and morphology of CaOx crystals differed between the tissues and organs of these species. In *Inula graveolens*, druses (diam. 10.00±1.71 μm) were observed in stem pith cells (Fig. 1) at ~5% frequency in this tissue. No crystals were found in epidermis or cortex cells of the stem. Leaf mesophyll cells of *I. graveolens* had druses (diam. 3.46±0.37 μm) (Fig. 2). No crystals were observed in leaf epidermal cells. Styloids (length 4.32±0.69 μm) appeared singly or clustered in each cell of the anther endothecial layers (Fig. 3). Almost every style cell had a single druse crystal (diam. 3.66±0.55 μm.) (Fig. 4). Each ovary cell had one



Figs. 1–6. Calcium oxalate crystals in *Inula graveolens*. **Fig. 1.** Druse crystal in stem pith cells. **Fig. 2.** Druse crystal in leaf mesophyll cells (arrow). **Fig. 3.** Styloids in anther endothecial cells (arrows). **Fig. 4.** Druse crystals in style cells. **Fig. 5.** Large prismatic crystals in ovary cells. **Fig. 6.** Styloids in head cells of glandular trichomes of the ovary (arrows). Bars = 10 μm. et – endothecial thickness.

large prismatic crystal (length 25.96±4.64 μm) (Fig. 5). Small styloids (length 3.17±0.91 μm) were also seen in the head cells of glandular trichomes of the ovaries (Fig. 6). No crystals were found in the filament or corolla cells of *I. graveolens*.

Pulicaria dysenterica had styloids (length 11.55±2.75 μm) in the endothecial layers of anthers (Fig. 7), as in *I. graveolens*. Druses (diam. 3.92±0.47 μm) were observed in the filaments of this species (Fig. 8). Druses (diam. 3.90±0.60 μm) were also present in the style cells (Fig. 9). Almost every style and filament cell had a single druse crystal. Every epidermal cell of the ovaries had one large prismatic crystal (length 24.67±2.35 μm) (Fig. 10). No crystals were seen in the stem, leaf or corolla cells of *P. dysenterica*.



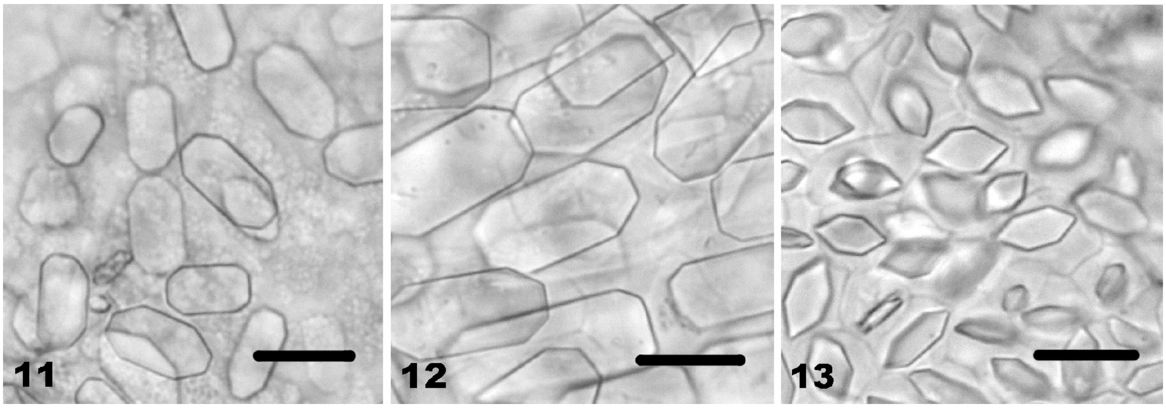
Figs. 7–10. Calcium oxalate crystals in *Pulicaria dysenterica*. **Fig. 7.** Styloids in endothecial cells of anthers (arrows) (stained with rubeanic acid). **Fig. 8.** Druses in filament cells (brightfield). **Fig. 9.** Druses in style cells (polarized light). **Fig. 10.** Large prismatic crystals in ovary cells (brightfield). Bar = 10 μm in Figs. 7, 8, 10, and 50 μm in Fig. 9. et – endothecial thickness.

Filago eriocephala, *Logfia arvensis* and *L. gallica* had prismatic crystals only in the ovary cells. These crystals were 12.06±1.96 μm long in *F. eriocephala* (Fig. 11), 14.32±1.28 μm long in *L. arvensis* (Fig. 12) and 8.70±1.14 μm long in *L. gallica* (Fig. 13). No CaOx crystals were observed in the other tissues of these three species. The crystals found were also confirmed to be CaOx histochemically with silver nitrate and rubeanic acid (Yasue, 1969). They stained brownish black with this technique.

DISCUSSION

Calcium oxalate (CaOx) crystals are found at all taxonomic levels in photosynthetic organisms, from small algae to higher plants (Franceschi and Nakata, 2005). The crystals are formed from endogenously synthesized oxalic acid and Ca taken from the environment, and they are produced and accumulated in species-specific morphologies. Several studies have described CaOx crystals in Asteraceae (Dormer, 1961; Horner, 1977; Meric and Dane, 2004; Meric, 2008). The present study examined the morphology and location of CaOx crystals in the Inuleae tribe. Crystals in this tribe displayed three types of morphology. Druses were observed in stem, leaf, filament and style cells. Styloids were seen only in anther endothecial cells, while prismatic crystals were found in the epidermis cells of ovaries. *Inula graveolens* differs remarkably from the other investigated species. It showed crystals in the stem, leaf, anther, style and ovary. *Filago eriocephala*, *Logfia arvensis* and *L. gallica* had crystals only in ovary epidermis cells. *Pulicaria dysenterica* displayed crystals in the anther, filament, style and ovary.

In the tribe Heliantheae, CaOx crystals have been investigated in the floral organs of the genus *Helianthus* (Horner, 1977; Meric and Dane, 2004). *H. annuus* contains prismatic and styloid crystals in the corollas (Meric and Dane, 2004), as well as styloid crystals in endothecial and tapetal cells of the anther (Horner, 1977; Meric and Dane, 2004). Styloids and prismatics are present in the filament. No crystals are seen in the ovary, although the style



Figs. 11–13. Calcium oxalate crystals in *Filago eriocephala*, *Logfia arvensis* and *L. gallica*. **Fig. 11.** Prismatic crystals in ovary cells of *F. eriocephala*. **Fig. 12.** Prismatic crystals in ovary cells of *L. arvensis*. **Fig. 13.** Prismatic crystals in ovary cells of *L. gallica*. Bar = 10 μm.

and stigma have styloid and druse crystals (Meric and Dane, 2004). *H. tuberosus* displays a pattern similar to that of *H. annuus* (Meric and Dane, 2004).

In the tribe Astereae, *Conyza* spp. are found to contain CaOx druse crystals in the stem, leaf and corolla cells (Meric 2008). No crystals are found in anthers or filaments. Ovary cells have styloids, while style cells contain druse crystals (Meric, 2008). These tribes therefore differ in their crystal patterns. The genus *Conyza* (Astereae) contains styloid crystals in the ovary, while in the Inuleae they are prismatic in ovary cells. No crystals are found in ovary cells of *Helianthus* spp. (Heliantheae). Styloid and prismatic crystals are present in the corolla of *Helianthus* species, while in *Conyza* species these are druses. No crystals were observed in the corolla cells of the Inuleae species studied here.

Crystals are not as common in herbaceous plant stems as in woody plant stems (Franceschi and Horner, 1980; Hudgins et al., 2003). It can be suggested that the large druses in stem pith cells of *I. graveolens* provide a storage site for Ca within the cell. No crystals were observed in the stem tissues of the other species examined. This is not surprising, as crystals are more common in leaves than in stems (Franceschi and Horner, 1980). Druses were only present in the mesophyll cells of the leaves, which make up the primary photosynthetic tissue in *I. graveolens*. Kuo-Huang et al. (2007) indicated that they are involved in dispersing light to the chloroplasts in the photosynthetic parenchyma cells of leaves. No crystals were observed in the leaves of the other investigated species. The presence of crystals in transitory floral organs such as the filament, anther and style is surprising. The function of crystals in these organs devoid of supporting tissues may be to provide strength to the tissues of these floral organs, which are critical to pollination and fertilization for sexual reproduction.

The ovaries of Asteraceae taxa are very interesting in respect to crystal size and shape. They have various prismatic crystals differing in size and morphology (Dormer 1961). Dormer (1961) reported prismatic crystal length ranging from 12 μm to 45 μm in seven species of Asteraceae. All the species in the present study also had prismatic crystals in their ovaries, with lengths varying from 8.70 μm to 25.96 μm depending on the species. Carpels and ovules are rich in cytoplasm, and maturing seeds are rich in nutrients. These crystals are very important in protecting against attacks by herbivores and/or strengthening tissues during seed maturation.

The morphology and distribution of crystals is constant within a species. This indicates that their presence, morphology and distribution in a species are under genetic control (Ilarslan et al., 2001; Franceschi and Nakata, 2005). Thus the constancy of crystal type and distribution may be considered a taxonomic character for classification of species (Franceschi and Nakata, 2005). The crystal pattern is also often stable within a genus (Lersten and Horner, 2000). This study was aimed at determining similarities and differences between taxa within the tribe. In the tissues and organs of the studied species of the Inuleae tribe, the crystal types were quite similar, although their distribution and presence or absence in tissues and organs differed. All the investigated species of the tribe Inuleae contained prismatic crystals in ovary epidermis cells and had no crystals in corolla cells. Further research will shed light on the taxonomical significance of crystals as an anatomical feature.

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