Identification of intracellular calcium oxalate crystals in

*Chamelaucium uncinatum* (Myrtaceae)

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Suggested running head title: Intracellular crystals in *Chamelaucium*
*Abstract.* Intracellular inclusions in the pedicel and calyx tube tissues of *Chamelaucium uncinatum* Schauer (Myrtaceae) flowers are irregular in shape. They were shown, using polarised light and scanning electron microscopy, to be birefringent 8.9-29.5 µm druse (i.e. aggregate) crystals. Energy-dispersive x-ray spectroscopy showed that these crystals were predominantly composed of calcium. Histochemical and acid solubility tests indicated that the crystals were calcium oxalate. Raman microprobe spectroscopy was used to confirm this chemical identity. The calcium oxalate crystals were located in xylem vessel lumens and also in parenchyma cells adjacent to vascular tissues. Thus, the crystals may function to regulate soluble calcium concentrations in *C. uncinatum* tissues near sites where calcium is unloaded from the xylem.

*Introduction*

Calcium (Ca) salts of oxalate, carbonate and phosphate are common crystalline mineral deposits in plants (Arnott and Pautard 1970). Ca oxalate (CaOx) is the most widely distributed inorganic crystal, occurring in 215 families and in organs such as roots, leaves, floral structures and seeds (Franceschi and Horner 1980; Webb and Arnott 1982; Prychid and Rudall 1999). CaOx crystals often form within the vacuoles of specialised idioblast cells in epidermal, ground and/or vascular tissues (Foster 1956; Franceschi and Horner 1980; Webb 1999). Crystals have distinctive morphologies including acicular or needle-shaped (i.e. raphide), spherical aggregates (i.e. druse), and prismatic (Franceschi and Horner 1980).
The functional significance of CaOx crystals in plants may vary according to crystal morphology and their distribution within tissues of a particular species (Franceschi and Horner 1980; Prychid and Rudall 1999). For instance, the sharp points of raphide crystals may protect tissue against foraging herbivores (Ward et al. 1997). Prismatic and druse crystals in cells surrounding vascular tissues may either contribute to the strength of tissue (Okoli and McEuen 1986) or act as a storage depository that regulates the concentration of soluble Ca and/or oxalate (Sunell and Healey 1979; Franceschi and Horner 1980).

Cellular inclusions also occur in members of the Myrtaceae (e.g. Eucalyptus erythrocorys seeds; Buttrose and Lott 1978). Geraldton waxflower (Chamelaucium uncinatum) Schauer (Myrtaceae) is a winter-to-spring flowering plant native to the south-west of Western Australia (Elliot and Jones 1984). In this species, flowers are borne on pedicels on short axillary shoots and develop from tight buds enclosed in bracteoles into open flowers with 5 waxy petals (Slater and Beardsell 1991; Olley et al. 1996). Chamelaucium uncinatum is widely cultivated as a cut flower crop and is Australia’s most valuable cut flower export (Joyce 1993). Cut flowering stems are exported to markets in the USA, Japan and Europe. Both in Australia and overseas, stems are used predominantly as a filler in floral arrangements owing to their relatively small flowers and leaves. Abscission of floral organs from harvested stems of C. uncinatum is a major problem that often limits marketability (Joyce 1993).

In an anatomical study of the floral organ abscission zone of C. uncinatum, we observed irregularly-shaped inclusions in adjacent pedicel and calyx tube cells. Thin
sectioning for ultrastructure investigation was impaired by these inclusions. The sections tore and the glass knife was damaged. Taylor (1999) observed similar inclusions in the mesophyll cells of *C. uncinatum* leaves. The current study characterises the distribution, morphology and chemical nature of intracellular inclusions in *C. uncinatum* pedicel and calyx tube tissues towards elucidating their biological role.

**Materials and methods**

*Plant material*

Investigations were performed on 6-8-year-old clonally-propagated *C. uncinatum* plants at commercial flower farms near Gatton (27° 34’ S, 152° 17’ E) (cvv. ‘Purple Pride’ and ‘Fortune Cookie’) and Crows Nest (27° 16’ S, 152° 03’ E) (cv. ‘Paddy’s Late’) in south-east Queensland, Australia. The cvv. ‘Purple Pride’, ‘Fortune Cookie’ and ‘Paddy’s Late’ are examples of early, mid and late flowering genotypes of *C. uncinatum*, respectively. During the growing season (October to March), plants were fertilised once every four weeks with 7 g N, 3 g K, 1.6 g P and 1.6 g Ca as urea, KNO₃, KPO₄ and CaNO₃, depending upon the particular farm. Plants at the Gatton farm were grown in a sandy loam soil that contained 487 mg kg⁻¹ Ca. At the Crows Nest farm, the predominant soil was granitic sand. Flowering stems were harvested from plants between June and October 2001. Fully open flowers with a pink nectiferous hypanthium (i.e. development stage 7; Olley *et al*. 1996) with attached pedicels were then removed from stems.
**Experimental**

Thirty flowers were removed at random from harvested stems of each *C. uncinatum* cultivar. Initially, eight replicate cv. ‘Purple Pride’ flowers were examined with light microscopy. However, because there was little variation in the morphology and distribution of inclusions in these flowers, only three replicate flowers from each cultivar were used for subsequent light, polarised light and fluorescence microscopy.

Five flowers were sampled for scanning electron microscopy and two flowers were used for energy-dispersive x-ray spectroscopy. Sections from a single flower were examined with Raman microprobe spectroscopy. Data on inclusion width and distribution density in tissues are presented as the mean ± standard error. Inclusion distribution density data were analysed by one-way ANOVA using Minitab™ (Release 13.1, Minitab Inc., State College, PA, USA) software. The LSD test at $P = 0.05$ was used to separate data means.

**Light microscopy**

Tissue segments comprising approximately 2 mm square blocks of pedicel and calyx tube tissues were excised either side of the abscission zone. These explants were fixed in 3% (v/v) glutaraldehyde (electron microscopy grade; ProSciTech, Thuringowa Central, Qld, Australia) in 0.1 M phosphate buffer (pH 7.2) for 14 h at 20°C. To prevent leaching of phenolics from the cytoplasm, 1% (w/v) anhydrous caffeine (Sigma Chemical Co., St Louis, MO, USA) was added to the fixative solution (Mueller and...
Greenwood 1978). The fixative was initially infiltrated for 10-20 min into the explants under reduced pressure of 93 kPa. Fixed explants were rinsed in three 10 min changes of the same 0.1 M phosphate buffer described above and dehydrated in a graded series of 5, 10, 15, 20, 25, 30, 40, 50, 60, 70, 80, 90, 100% (v/v) acetone for 45 min at each step. Each concentration of acetone was initially infiltrated into tissue for 10 min at 93 kPa. The dehydrated explants were then infiltrated with Spurr’s low viscosity epoxy resin (Spurr’s embedding kit, ProSciTech, Thuringowa Central, Qld, Australia; Spurr 1969) in graded concentrations, with 12 h each in 50, 75 and 100% resin in acetone, followed by five 12 h changes in 100% resin. Infiltrated explants were embedded into fresh Spurr’s resin and cured for 3 days at 60°C.

Longitudinal and transverse tissue sections 0.5 μm-thick through abscission zones were cut using a glass knife on a Reichert-Jung Ultracut E microtome (Reichert-Jung, Austria). The sections were placed onto a drop of distilled water on microscope slides, heated at 70°C, and stained with toluidine blue O [0.5% (w/v) toluidine blue in 1% (w/v) borax; Sigma-Aldrich Pty Ltd, Castle Hill, NSW, Australia] for 10 s at 70°C (O’Brien and McCully 1981). Sections were washed with distilled water and viewed under bright field illumination with an Olympus BH-2 light microscope (Olympus Optical Co., Ltd, Tokyo, Japan). The sections were photographed using an Olympus PM-10AK photomicrographic system with Kodak 200 ISO colour film. Unstained sections were examined under polarised light using an Olympus BH-2-UMA light microscope fitted with Olympus UPO (NIC) polarising filters. Images of these sections were taken in digital form using Cool SNAP imaging software (Roper Scientific Inc., Tucson, AZ, USA). Unstained sections were also viewed under ultra violet (UV)
illumination (HBO 100) using an Axioskop 20 (Carl Zeiss Co., Germany) light microscope fitted with fluorescence filters (filter set 05, Carl Zeiss Co., Germany) and photographed with an Olympus OM-4Ti camera using Kodak 400 ISO Ektachrome colour film. This UV system allowed for excitation with 395 to 440 nm light and restricted emission to wavelengths of > 470 nm. Final magnifications on all photomicrographs were determined from photomicrographs of a stage micrometer.

The chemical nature of inclusions in the tissue sections was investigated using an oxalate histochemical staining method (Yasue 1969). Sections on microscope slides were treated with 5% (v/v) glacial acetic acid (BDH Laboratory Supplies, Poole, England) for 30 min to dissolve carbonate and phosphate salts, washed with distilled water, and immersed into 5% (w/v) aqueous silver nitrate (analytical grade; Ajax Chemicals, Auburn, NSW, Australia) for 15 min. Sections were then washed with distilled water and treated with saturated dithio-oxamide (98% purity; Aldrich Chemical Co., Milwaukee, WI, USA) in 70% (v/v) ethanol containing two drops of 28% ammonia solution (analytical grade, present as ammonium hydroxide; Ajax Chemicals, Auburn, NSW, Australia)/100mL for 1 min, followed by a brief wash in 50% (v/v) ethanol and a rinse in distilled water. The sections were then counterstained with toluidine blue O as described above. Oxalate stains black in the Yasue (1969) procedure. Other unstained sections were treated with 2% (v/v) hydrochloric acid (specific gravity 1.16; BDH Laboratory Supplies, Poole, England) for 30 min. Oxalate readily dissolves in hydrochloric acid (Yasue 1969). The presence of carbonates was assessed in other sections by treatment with 1% (w/v) alizarin red S (alizarin sulphonate sodium; George T. Gurr Ltd, London, England) for 3 min at 70°C, whereby carbonates stain red.
(Johnson and Pani 1962). All reagent-treated sections were viewed with bright field and polarised light microscopy.

**Scanning electron microscopy**

Tissue explants from the cv. ‘Purple Pride’ excised and fixed as described above were also used for scanning electron microscopy. Fixed explants were washed in three changes of 0.1 M phosphate buffer as described above, post-fixed in 1% (v/v) osmium tetroxide (spectroscopy grade; ProSciTech, Thuringowa Central, Qld, Australia) in 0.1 M sodium cacodylate buffer (O'Brien and McCully 1981) for 2 h at 20°C, and washed again in 0.1 M phosphate buffer. Explants were frozen in liquid nitrogen and fractured apart by lightly tapping the tissue with a cooled razor blade. Fractured explants were then freeze-dried for 3 days at -35°C. Longitudinally fractured pieces of pedicel tissues were mounted onto aluminium stubs and coated with gold using a SPI Supplies coating unit (SPI Supplies, West Chester, PA, USA). Fracture surfaces and intracellular inclusions thereon were examined with a JEOL JSF6300 field emission scanning electron microscope (JEOL Ltd, Tokyo, Japan) at 8 kV. Images of tissues were taken in digital form using Image Slave software (OED Pty Ltd, Hornsby, NSW, Australia).

**Energy-dispersive x-ray spectroscopy**

The relative elemental composition of longitudinally fractured pedicel tissues was determined on excised explants prepared as described for scanning electron microscopy. A JEOL JSF6400 field emission scanning electron microscope fitted with an Oxford Si-
Li energy-dispersive spectrometer (Oxford Instruments Plc., High Wycombe, United Kingdom) was used. The 20 kV electron beam was focused on the centre of intracellular inclusions that were 20-25 µm wide. Corresponding points in cells that did not contain inclusions were also analysed as controls. Scatter from the electron beam on contact with the inclusions and tissues was approximately 10 µm in diameter. A Moran Scientific multi-channel analyser (Moran Scientific Pty Ltd, Goulburn NSW, Australia) was used to examine the emission of x-ray spectra from samples, which were generated from a 100 s live-time analysis.

Raman microprobe spectroscopy

The chemical identity of intracellular inclusions was confirmed using Raman microprobe spectroscopy (Frost et al. 2002). Pedicel tissue explants from each cultivar were fixed, dehydrated, embedded and sectioned as described above for light microscopy. Inclusions in unstained longitudinal sections mounted on glass microscope slides were viewed under an Olympus BHSM light microscope connected to a Renishaw RM1000 Raman microscope system (Renishaw Plc., Wotton-under-Edge, United Kingdom). To obtain the Raman spectra, inclusions were excited by a Spectra-Physics model 127 He-Ne laser at 633 nm (Spectra-Physics Inc., Mountain View, CA, USA) with a resolution of 2 cm⁻¹ between 100 and 4000 cm⁻¹. Repeated scans were run to increase the signal to noise ratio in spectra. Raman spectra were also generated on a sample of analytical grade CaOx monohydrate form (Ajax Chemicals, Auburn, NSW, Australia). Spectra were calibrated against a silicon wafer line at 520.5 cm⁻¹.
Results

All cell walls in longitudinal sections through the central axes of *C. uncinatum* cv. ‘Purple Pride’ pedicels and calyx tubes (Fig. 1A) stained blue with toluidine blue O. Intracellular inclusions were observed at higher magnification in pedicel and calyx tube tissues (Fig. 1B). They were light brown in toluidine blue O stained sections, and were predominant in parenchyma cells adjacent to pedicel and calyx tube vascular tissues. The inclusions were irregular in shape (Fig. 1C). Only the outlines of inclusions in parenchyma cells were observed in some locations, presumably because the inclusions themselves were torn out during sectioning. They were hard and damaged glass knives during sectioning, thereby resulting in scratched and torn sections. Widths of inclusions ranged from 5.8-28.8 µm (*n* = 32).

Insert Fig. 1 about here

The distribution density of inclusion-containing cells in pedicels was highest within 100 µm of vascular tissues and decreased with increasing distance from the vascular tissues (Fig. 1D, Table 1). At higher magnification, the distribution of inclusion-containing cells near vascular tissues in pedicels was variable, in that parenchyma cells adjacent to inclusion-containing cells frequently did not contain inclusions (Fig. 1E). A similar density and distribution pattern was observed in calyx tubes (Fig. 1F). Inclusions were also observed inside xylem vessels in both pedicels and calyx tubes (Fig. 1E, F). Cells containing inclusions did not appear to be visibly different in either size or shape from adjacent cells that did not contain inclusions (Fig.
Likewise, inclusions were observed in pedicel and calyx tube xylem and parenchyma cells of cvv. ‘Fortune Cookie’ and ‘Paddy’s Late’ (not shown). In all cultivars, inclusions were of the same irregular shape and had the same irregular distribution pattern.

Inclusions in longitudinal sections through pedicel and calyx tube tissues were birefringent under polarised light (Fig. 2A). That is, they gave bright refraction of light relative to surrounding tissue; which is a characteristic of crystalline deposits (Frey-Wyssling 1981). The crystals also autofluoresced at wavelengths > 470 nm when exposed to near UV light of 395-440 nm (Fig. 2B).

Observation of longitudinally fractured pedicels by scanning electron microscopy confirmed the presence of crystals in cells (Fig. 2C). Crystals were of a druse (i.e. aggregate) habit, ranging from 8.9-29.5 µm in width (n = 10). Some of the larger crystals occupied the entire cell lumen. Evidence of a crystal sheath (Webb and Arnott 1981) was seen surrounding some crystal facets, where it was apparently torn off during fracturing (Fig. 2C).

Crystals stained black upon treatment with a silver nitrate-rubeanic acid sequence. This reaction indicated the presence of oxalate, carbonate or phosphate salts in crystals (Fig. 2D). Crystals readily dissolved without observable effervescence when treated with 2% hydrochloric acid. When pre-treated with 5% acetic acid for 30 min,
most crystals dissolved without effervescence, leaving behind an outline of the crystal shape in cells (Fig. 2E). When subsequently stained with the silver nitrate-rubeanic acid test, crystals could not be seen under bright field microscopy. However, under polarised light, it was evident that some crystals did not completely dissolve in acetic acid (Fig. 2F). Complete dissolution in hydrochloric acid and incomplete dissolution in acetic acid suggests that crystals were composed of oxalate. Crystals were apparently not composed of carbonate, because they did not stain red when treated with alizarin red S.

Insert Fig. 2 about here

Elemental analysis of crystals in fractured C. uncinatum cv. ‘Purple Pride’ pedicels by energy-dispersive x-ray spectroscopy confirmed that Ca was the dominant element present based on a Ca peak:background ratio of 62.8:1. In contrast, the lumen of cells in which no crystals were present only contained trace concentrations of Ca and K with peak:background ratios of 8.4:1 and 15.4:1, respectively.

Raman spectra in the range of 400-1800 cm\(^{-1}\) of crystals from each of the three C. uncinatum cultivars (Fig. 3A, B, C) were very similar to spectral bands for the CaOx reference sample (Fig. 3D). Bands at 898/894, 1464/1462 and 1490/1488 cm\(^{-1}\) in crystals and equivalent bands at 898, 1464 and 1492 cm\(^{-1}\) in the CaOx reference sample indicated that C. uncinatum crystals were composed of CaOx. Raman spectral bands for pure CaOx monohydrate occur at 894, 1462 and 1488 cm\(^{-1}\) while pure CaOx dihydrate is characterised by bands at 911, 1478 and 1634 cm\(^{-1}\) (Kontoyannis et al.)
The similar patterns in Raman spectral bands for *C. uncinatum* crystals and those published for pure CaOx monohydrate suggests that the druse crystals were CaOx monohydrate.

Discussion

Intracellular inclusions in *C. uncinatum* were identified as aggregate (i.e. druse) CaOx monohydrate crystals by a sequence of optical and chemical analyses (Fig. 2A, C, D, F), including Raman microprobe spectroscopy (Fig. 3). As far as the authors are aware, this is the first reported use of Raman spectroscopy to characterise plant crystals *in situ*. Ward *et al.* (1979) used Raman spectroscopy to determine the chemical identity and fate of crystals from *Medicago sativa* L. leaves after digestion by cattle. Raman spectroscopy is used in biomedical (Lawson *et al.* 1997) and materials (Lyon *et al.* 1998) science. The current study shows Raman microprobe spectroscopy has applied potential to determine the chemical identity of crystals in plants where they cannot feasibly be extracted for x-ray diffraction (Al-Rais *et al.* 1971) or infrared spectroscopy (Scurfield *et al.* 1973; Horner and Zindler-Frank 1982).

CaOx crystals are common plant cell inclusions, occurring in 215 families including the Myrtaceae of which *C. uncinatum* is a member (Franceschini and Horner 1980; Prychid and Rudall 1999). We identified for the first time CaOx crystals in *Chamelaucium uncinatum* (Myrtaceae). CaOx crystals have been reported in other
native Australian genera. For example, crystals were found in seed protein bodies of *Eucalyptus erythrocorys* (Myrtaceae) and *Macadamia integrifolia* (Proteaceae) (Buttrose and Lott 1978; Lott and Buttrose 1978a), and also in cell walls of branchlets of *Allocasuarina* sp., *Casuarina* sp., and *Gymnostoma papuanum* (Casuarinacea) (Berg 1994). Crystal-like inclusions have also been seen in both the anther and cells surrounding the ovary of the flowers and fruits of *Thryptomene calycina* (Myrtaceae) (Beardsell *et al.* 1993), and in *C. uncinatum* leaf mesophyll cells (Taylor 1999).

CaOx crystals form within epidermal, ground (e.g. parenchyma, mesophyll) and vascular tissues (e.g. phloem, xylem, bundle sheath cells) in a range of plant organs, including roots, stems, leaves, floral structures and seeds (Franceschi and Horner 1980; Webb 1999). In *C. uncinatum* pedicels and calyx tubes, druse CaOx crystals were found in xylem vessels and in parenchyma cells adjacent to vascular tissues. The distribution density of crystal-containing cells was highest within 100 µm distance from vascular tissues (Fig. 1D, Table 1). This finding is consistent with observations of Sunell and Healey (1979), who reported that the density of druse CaOx crystals in taro (*Colocasia esculenta* L.) corms was highest in tissue surrounding vascular bundles. Druse CaOx crystals in the leaves of *Hibiscus esculentus* L., *Gleditsia triacanthos* L., *Telfairia* spp. Hook., and *Prunus* spp. L. are also predominantly distributed within vascular bundles and in adjacent mesophyll and parenchyma tissues (Webb and Arnott 1981; Borchert 1984; Okoli and McEuen 1986; Lersten and Horner 2000). In addition, in *C. uncinatum* pedicel and calyx tube tissues, CaOx crystal-containing cells did not visibly differ in either size or shape from neighbouring cells that did not contain crystals (Fig. 1E, F). CaOx crystals commonly form intracellularly in plants, typically within
vacuoles of specialised crystal idioblast cells (Franceschi and Horner 1980). Such cells can be readily differentiated because they are usually larger in size and possess a more densely-packed cytoplasm rich in organelles and vesicles than adjacent non-crystal forming cells (Franceschi and Horner 1980; Webb 1999).

The function of CaOx crystals in plants is not completely understood, and probably varies according to crystal morphology and their distribution (Franceschi and Horner 1980; Prychid and Rudall 1999). Acicular (needle-like) CaOx raphide crystals may help protect plant tissue against foraging herbivores. When tissues (e.g. leaves) containing these crystals are eaten, the sharp points of crystals can injure mouth and throat tissues (Gardner 1994; Ward et al. 1997). Prismatic and aggregate CaOx crystals located in cells surrounding vascular tissues have been suggested to either provide additional structural support to plant tissue (Okoli and McEuen 1986) or act as a storage mechanism for Ca, oxalate and toxic metals (e.g. barium) (Lott and Buttrose 1978b; Sunell and Healey 1979; Franceschi and Horner 1980).

Formation of CaOx crystals in vacuoles of crystal idioblast cells has also been correlated with increasing Ca supply and tissue concentrations in plants such as *Phaseolus vulgaris* L. (Zindler-Frank 1995; Zindler-Frank et al. 2001). Thus, plants may form CaOx crystals to remove excess soluble Ca from the cytoplasm (Borchert 1986; Webb 1999), and, perhaps, regulate the supply of Ca during periods of deficiency in order to maintain optimal soluble Ca concentrations in tissue (Franceschi and Horner 1979; Franceschi 1989). Accordingly, we suggest that CaOx crystals may function to reduce the cytoplasmic Ca concentration in *C. uncinatum*. This proposal is consistent
with the findings of Taylor (1999) that *C. uncinatum* plants exhibit conservative allocation of calcium. That is, they can grow at a range of Ca nutrition rates without developing either visible deficiency or toxicity symptoms. Furthermore, based on the distribution of crystals in *C. uncinatum* xylem vessels and in parenchyma cells adjacent to vascular tissues, the druse CaOx crystals may regulate soluble Ca concentrations in tissues near where this element is unloaded from the xylem.

A key question is how might CaOx accumulation enable *C. uncinatum* to adapt to its natural environment. *Chamelaucium uncinatum* is endemic to the south-west of Western Australia (*c.* 27° 40’S, 114° 26’S to 31° 57’S, 115° 51’E) (Manning *et al*. 1996). The soils in this region are predominantly either deep white to yellow sands or shallow white sands overlying limestone that are typically low in nutrients (Northcote *et al*. 1975; Bettenay 1993; Egerton-Warburton *et al*. 1995). For example, these soils were found to contain 0.2-2.7% organic carbon, 1-17 mg kg\(^{-1}\) P, 1-7 mg kg\(^{-1}\) NO\(_3\), 1-8 mg kg\(^{-1}\) NH\(_4\) and 20-150 mg kg\(^{-1}\) K (Egerton-Warburton *et al*. 1995). While no data on the Ca concentrations in these soils is presently available (D. Growns, pers. comm.), we suggest that *C. uncinatum* plants may have evolved mechanisms for acquiring adequate Ca from soils low in Ca and for tolerating periods of high Ca supply in other circumstances (Taylor 1999).

In summary, this study has shown that *C. uncinatum* accumulates Ca in the form of druse intracellular CaOx crystals in both pedicel and calyx tube xylem and parenchyma cells. Further research directed at understanding environmental and cellular cues
controlling the genesis of these CaOx crystals is required to establish their functional significance.

Acknowledgments

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References


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Table 1. Distribution density of inclusion-containing cells in pedicels of *Chamelaucium uncinatum* cv. ‘Purple Pride’ for 0-100, 100-200, 200-300 and 300-400 µm radii from the outer edge of central vascular tissues (i.e. phloem)

Values (means ± s.e.m., *n* = 3 individuals) followed by different letters are significantly different at *P* = 0.05

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<tr>
<th>Distance from vascular tissues (µm)</th>
<th>Distribution density (number of cells µm⁻²)</th>
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<tr>
<td>0 – 100</td>
<td>28.7 x 10⁻⁵ ± 5.1 x 10⁻⁵ a</td>
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<tr>
<td>100 – 200</td>
<td>11.8 x 10⁻⁵ ± 1.3 x 10⁻⁵ b</td>
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<td>200 – 300</td>
<td>6.2 x 10⁻⁵ ± 0.6 x 10⁻⁵ bc</td>
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<td>300 – 400</td>
<td>1.1 x 10⁻⁵ ± 0.5 x 10⁻⁵ c</td>
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List of Figure captions

Fig. 1. Light micrograph sections through *C. uncinatum* cv. ‘Purple Pride’ pedicel and calyx tube tissues of containing intracellular inclusions. Longitudinal section through pedicel and calyx tube tissues at 40 X (A) and 100 X (B) magnification. Longitudinal section through pedicel tissues at 400 X magnification (C). Transverse section through pedicel tissues at 100 X (D) and 400 X (E) magnification. Transverse section through calyx tube tissues at 400 X magnification (F). Scale bars represent 500 µm in A, 100 µm in B and D, and 50 µm in C, E and F. Pedicel and calyx tube tissues were fixed in glutaraldehyde, dehydrated in acetone and embedded in Spurr’s resin. All sections were cut to 0.5 µm thick and stained with toluidine blue O. B = bracteole attachment point, C = calyx tube, I = intracellular inclusion, O = oil gland, Pa = parenchyma, Pe = pedicel, Ph = phloem, V = vascular tissues, X = xylem. The arrows in C point to typical inclusions in which the centre is missing. The arrows in E and F point to inclusions inside xylem vessels.

Fig. 2. Light and scanning electron micrographs showing sectioned and fractured *C. uncinatum* cv. ‘Purple Pride’ pedicel and calyx tube tissues containing intracellular crystal inclusions. Longitudinal section through pedicel and calyx tube tissues under polarised light at 200 X magnification (A) and near-UV illumination at 100 X magnification (B). Longitudinal fracture of pedicel parenchyma cell containing druse crystal viewed by scanning electron microscopy at 8 kV and 3000 X magnification (C). Longitudinal section through pedicel tissues viewed at 400 X magnification showing dark staining (D) and dissolution of crystals (E). Longitudinal section through pedicel
tissues under polarised light showing partial dissolution of crystals at 400 X magnification (F). Pedicel and calyx tube tissues for light microscopy were fixed in glutaraldehyde, dehydrated in acetone and embedded in Spurr’s resin. All sections were cut to 0.5 μm thick. Sections in D and E were stained with toluidine blue O. Pedicel tissues for scanning electron microscopy were fixed as described above for light microscopy, post-fixed in osmium tetroxide, freeze-fractioned apart in liquid nitrogen and sputter-coated with gold. Scale bars represent 100 μm in A and B, 10 μm in C, and 50 μm in D, E and F. C = calyx tube, CI = crystal inclusion, I = inclusion, O = oil gland, Pe = pedicel. The arrow in C points to remnants of a sheath around crystal facets.

**Fig. 3.** Energy-dispersive x-ray spectra for elements in crystals (A) and lumen tissues (B) in *C. uncinatum* cv. 'Purple Pride' pedicel parenchyma cells. Pedicel tissues were fixed in glutaraldehyde, post-fixed in osmium tetroxide, dehydrated in acetone and freeze-fractioned apart in liquid nitrogen and sputter-coated with gold for analysis. The gold peaks are artifacts due to sputter-coating. Spectra were generated from a 100 second spot analysis at an accelerating voltage of 20 kV.

**Fig. 4.** Raman spectra of intracellular crystals in 0.5 μm thick longitudinal sections through pedicel tissues of *C. uncinatum* cvv. 'Fortune Cookie' (A), 'Paddy's Late' (B) and 'Purple Pride' (C) and of a CaOx chemical standard (D). The frequencies of key spectral bands are indicated. The baseline of Raman spectra for (B), (C) and (D) have been shifted by a Raman intensity of 10 000, 20 000 and 40 000, respectively, relative to (A), for convenience of viewing.
Figure 1.
Figure 2.
Figure 3.
Figure 4.