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Identification of intracellular calcium oxalate crystals in *Chamelaucium uncinatum* (Myrtaceae)

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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 to 29.5 μm druse (i.e. aggregate) crystals. Energy-dispersive x-ray spectroscopy showed that crystals were predominantly composed of calcium. Histochemical and acid solubility tests indicated that crystals were calcium oxalate. Raman microprobe spectroscopy was used to confirm this chemical identity. The druse calcium oxalate crystals were located in xylem vessel lumens and in parenchyma cells adjacent to vascular tissues in *C. uncinatum* pedicels and calyx tubes. Based on this pattern of crystal distribution, the crystals may function to regulate soluble calcium concentrations in *C. uncinatum* tissues near sites where calcium is unloaded from the xylem.

Introduction

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). Flowers are borne on pedicels on short axillary shoots. They 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 (i.e. flower buds, open flowers) from their pedicels during postharvest handling is a major problem affecting the marketability of cut flowering *C. uncinatum* stems (Joyce 1993). Floral organs abscise at a morphologically distinct abscission zone located at the base of the calyx tube (Joyce 1993). In an anatomical study of this abscission zone, we observed irregularly-shaped inclusions in pedicel and calyx tube cells. Thin sectioning for ultrastructure investigation was impaired by these inclusions, in that the sections tore and the glass knife was damaged. Taylor (1999) observed similar inclusions in mesophyll cells of *C. uncinatum* leaves.

The current study investigated the distribution, morphology and chemical nature of intracellular inclusions in *C. uncinatum* pedicel and calyx tube tissues.

Materials and Methods

Plant material

Investigations were performed on 6 to 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. During the growing season (October to March), plants were fertilised once every four weeks with 7 g nitrogen, 3 g potassium (K), 1.6 g phosphorus (P) and 1.6 g calcium (Ca) as urea, K nitrate, K phosphate and Ca nitrate, 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.

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 to 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 d at 60°C.

Longitudinal and transverse 0.5 μm thick tissue sections 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 d 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 focussed to a spot on intracellular inclusions. Corresponding points in cells that did not contain inclusions were also analysed as controls. 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 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 Ca oxalate (CaOx) monohydrate form (Ajax Chemicals, Auburn, NSW, Australia). Spectra were calibrated against a silicon wafer line at 520.5 cm^{-1}.

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 means ± standard errors.

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 to 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 (Figs 1E and F). Cells containing inclusions did not appear to be visibly different in either size or shape from adjacent cells that did not contain inclusions (Figs 1C, E and F). 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.

Insert Table 1 about here

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 to 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 to 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). 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 the presence of Ca (Fig. 3A). In contrast, the lumen of cells in which no crystals were present only contained trace

concentrations of Ca (Fig. 3B). The presence of gold is an artifact of the sputter coating process.

Insert Fig. 3 about here

Raman spectra in the range of 400 to 1800 cm^{-1} of crystals from each of the three *C. uncinatum* cultivars (Figs 4A, B and C) were very similar to spectral bands for the CaOx reference sample (Fig 4D). 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 indeed composed of CaOx.

Insert Fig. 4 about here

Discussion

In the present study, intracellular inclusions in *C. uncinatum* were identified as CaOx crystals through a sequence of optical and chemical analyses. Inclusions were initially determined to be crystalline based on their bright birefringence of polarised light (Fig 2A; Frey-Wyssling 1981). These crystals were observed to autofluoresce when exposed to near-UV light (Fig. 2B). Use of fluorescence microscopy (O'Brien and McCully 1981) to identify crystals in plant tissue has not been reported. The specificity of fluorescence for identifying crystals would need to be extensively tested to determine reliability. The crystals were shown by scanning electron microscopy to be of an aggregate or druse type that sometimes occupied the entire cell lumen (Fig. 2C).

Energy-dispersive x-ray spectroscopy analysis of these druse crystals revealed that the principal element was Ca (Figs 3A and B). Druse Ca crystals stained positively for oxalate, carbonate and phosphate ions (Fig. 2D) when treated with the silver nitrate-rubeanic acid protocol (Yasue 1969).

Identification of the Ca crystal anion has commonly been based on solubility in various acids (Webb and Arnott 1982). Yasue (1969) showed that Ca anions of oxalate, carbonate and phosphate dissolved in 2% hydrochloric acid within 30 min, while only carbonate and phosphate completely dissolved within 30 min in 5% acetic acid. Incomplete dissolution of *C. uncinatum* crystals in 5% acetic acid over 30 min (Fig. 2F) plus their failure to stain in alizarin red S indicated that crystals were not Ca carbonate or phosphate. Confirmation of the crystals not being Ca phosphate was provided by energy-dispersive x-ray spectral analysis, in that P was not detected (Fig. 3A).

X-ray diffraction or infrared spectroscopy is often used to complement histochemical and acid solubility tests (Horner and Zindler-Frank 1982). Prior to analysis by x-ray diffraction or infrared spectroscopy, entire crystals generally have to be extracted from plant tissue (Al-Rais *et al*. 1971; Scurfield *et al*. 1973). However, for plant tissue in which crystals are small and/or sparsely distributed, their isolation in quantities sufficient for analysis is problemmatical (Horner and Zindler-Frank 1982). In view of their small size and infrequent distribution, Raman microprobe spectroscopy was used to determine the chemical identity of intact crystals in sectioned *C. uncinatum* tissue. This analysis confirmed that *C. uncinatum* crystals were composed of CaOx (Fig. 4).

As far as the authors are aware, this is the first reported use of Raman spectroscopy to characterise plant crystals *in situ*. However, Raman microprobe spectroscopy has been used to determine the chemical identity and fate of crystals from *Medicago sativa* L. leaves after digestion by cattle (Ward *et al*. 1979). Raman spectroscopy is used in biomedical (Lawson *et al*. 1997) and materials (Lyon *et al*. 1998) science. It has been used to determine the chemical identity of urinary calculi (stones) in humans (Kontoyannis *et al*. 1997). 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 or infrared spectroscopy.

CaOx crystals are common plant cell inclusions, occurring in 215 families (Franceschi and Horner 1980; Ward *et al*. 1997; Prychid and Rudall 1999). In the present study, druse CaOx crystals were identified for the first time in pedicel and calyx tube cells of the native Australian plant, *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 1978), and in cell walls of branchlets of *Allocasuarina* sp., *Casuarina* sp., and *Gymnostoma papuanum* (Casuarinacea) (Berg 1994). Crystal-like inclusions, possibly composed of CaOx, have also been seen in *Thryptomene calycina* (Myrtaceae) (D. Beardsell, pers. comm.) and in *C. uncinatum* leaf mesophyll cells (Taylor 1999).

As implied above, CaOx crystals occur in various plant tissues of a range of organs, including roots, stems, leaves, floral organs and seeds (Franceschi and Horner 1980).

These crystals are microscopic in size and commonly form intracellularly in plants; typically within vacuoles of specialised crystal idioblast cells (Franceschi and Horner 1980). Relative to neighbouring cells, crystal idioblast cells are usually larger in size and possess a more densely-packed cytoplasm that is rich in organelles and vesicles which surround crystal-forming vacuoles (Franceschi and Horner 1980; Webb 1999). In *C. uncinatum* pedicel and calyx tube tissues, CaOx crystals were present in xylem vessels and in parenchyma cells adjacent to vascular tissues (Figs 1E and F).

CaOx occurs in plants in monohydrate and dihydrate states (Frey-Wyssling 1981). Raman spectroscopy can be used to identify the hydration state of an unknown chemical by reference to a chemical of known hydration state. In a study of urinary stones in humans, Kontoyannis *et al*. (1997) determined that pure CaOx monohydrate produced Raman spectral bands at 894, 1462 and 1488 cm^{-1}. In contrast, pure CaOx dihydrate was characterised by bands at 911, 1478 and 1634 cm^{-1}. Raman spectral bands for CaOx druse crystals in each of the three *C. uncinatum* cultivars were at 894/898, 1462/1464 and 1488/1492 cm^{-1}. The similar patterns in Raman spectral bands for *C. uncinatum* crystals and those for pure CaOx monohydrate (e.g. Kontonyannis *et al*. 1997) suggests that the druse crystals were CaOx monohydrate. CaOx monohydrate is both more stable and more commonly found in plants than CaOx dihydrate (Frey-Wyssling 1981).

The function of CaOx crystals in plants is not completely understood, and probably varies according to crystal morphology and 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 the mouth and throat (Gardner 1994; Ward *et al*. 1997). Prismatic and aggregate CaOx crystals in cells surrounding vascular tissues have been suggested to provide additional structural support to plant tissue (Okoli and McEuen 1986). CaOx crystals may also regulate the concentration of soluble Ca in plant tissue (Franceschi and Horner 1980; Borchert 1986). Plants may use crystals to store excess Ca, and, perhaps, for supply during periods of deficiency (Franceschi and Horner 1979; Franceschi 1989).

Chamelaucium uncinatum is endemic to the south-west of Western Australia (ca. 27° 40'S, 114° 26'S to 31° 57'S, 115° 51'E) (Manning *et al*. 1996). The soils in this region are predominantly 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 to 2.7% organic carbon, 1 to 17 mg kg^{-1} P, 1 to 7 mg kg^{-1} nitrate, 1 to 8 mg kg^{-1} ammonium and 20 to 150 mg kg^{-1} K (Egerton-Warburton *et al*. 1995). No data on the Ca concentrations in these soils is presently available (D. Growns, pers. comm.). Accordingly, *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).

Formation of CaOx crystals in vacuoles of crystal idioblast cells has been correlated with increasing Ca supply and tissue concentrations in plants such as *Phaseolus vulgaris* L. (Zindler-Frank 1995; Zindler-Frank *et al*. 2001). CaOx crystals could

represent a mechanism by which plants remove excess soluble Ca from the cytoplasm (Borchert 1986; Webb 1999). The suggestion that CaOx crystals function to reduce cytoplasmic Ca concentration in *C. uncinatum* is consistent with the findings of Taylor (1999) that *C. uncinatum* plants can grow on a range of Ca nutrition rates without developing 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 crystals may regulate soluble Ca concentrations in tissues near where Ca is unloaded from the xylem.

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

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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 are the means ± s.e.m. (n = 3 individuals)

Distance from vascular tissues (μm)	Distribution density (number of cells μm^{-2})
0 – 100	$28.7 \times 10^{-5} \pm 5.1 \times 10^{-5}$
100 – 200	$11.8 \times 10^{-5} \pm 1.3 \times 10^{-5}$
200 – 300	$6.2 \times 10^{-5} \pm 0.6 \times 10^{-5}$
300 – 400	$1.1 \times 10^{-5} \pm 0.5 \times 10^{-5}$