Anatomy of ethylene-induced floral organ abscission in *Chamelaucium uncinatum* (Myrtaceae)

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Abstract. Postharvest abscission of Geraldton waxflower (*Chamelaucium uncinatum* Schauer) flower buds and flowers is ethylene-mediated. Exposure of floral organs to exogenous ethylene (1 μL L^{-1}) for 6 h at 20°C induced separation at a morphologically and anatomically distinct abscission zone between the pedicel and floral tube. Flower buds with opening petals and flowers with a nectiferous hypanthium were generally more responsive to exogenous ethylene than were flower buds enclosed in shiny bracteoles and aged (senescing) flowers. The anatomy of abscission zone cells did not change at sequential stages of floral development from immature buds to aged flowers. The zone comprised a layer of small, laterally elongated-to-rounded, closely packed and highly protoplasmic parenchyma cells. Abscission occurred at a two to four cell-wide separation layer within the abscission zone. The process involved degradation of the middle lamella between separation layer cells. Following abscission, cells on both the proximal and distal faces of the separation layer became spherical, loosely packed and contained degenerating protoplasm. Central vascular tissues within the surrounding band of separation layer cells became torn and fractured. For flower buds, bracteoles that enclose the immature floral tube also separated at an abscission zone. However, this secondary abscission zone appeared less sensitive to ethylene than the primary (central) floral tube abscission zone as bracteoles generally only completely abscised when exposed to 10 μL L^{-1} ethylene for the longer period of 24 h at 20°C. The smooth surfaces of abscised separation layer cells suggest that hydrolase enzymes degrade the middle lamella between adjacent cell walls.

Introduction

Abscission is the process of controlled shedding of plant organs, such as leaves, fruit and floral structures. It is a metabolic process that occurs in response to developmental, environmental and hormonal cues (Taylor and Whitelaw 2001). The principal hormonal regulators of abscission are ethylene and auxin, which accelerate and inhibit the process, respectively (Sexton *et al*. 1985). Ethylene accelerates floral organ abscission in a range of horticultural plants, including tomato (*Lycopersicon esculentum* L.), rose (*Rosa hybrida* L.), and snapdragon (*Antirrhinum majus* L.) (Roberts *et al*. 1984; van Doorn 2002). From a commercial perspective, premature floral organ abscission reduces the marketability of susceptible commodities, such as cut flowers and potted plants (Reid 1995).

Abscission usually occurs at morphologically and anatomically distinct abscission zones. Abscission zones can often be identified as a narrow constriction in tissue at the juncture between the plant body and subtending organ (Addicott 1982). They may also be recognised as a discrete plane of small, closely packed and highly protoplasmic cells (Sexton and Roberts 1982). Separation of abscission zone cells typically involves dissolution of the middle lamella and weakening of the primary cell wall in association with secretion of cell wall-degrading hydrolases (Roberts *et al*. 2002). Cells at the separation layer may then expand or round-up (Sexton and Redshaw 1981). Vascular tissues (e.g. xylem vessels) that traverse the abscission zone are not readily degraded by cell wall hydrolases. Rather, they tend to tear because of an increase in mechanical stress generated by expanding abscission zone cells (Sexton 1976; Sexton and Redshaw 1981).

Chamelaucium uncinatum Schauer (Myrtaceae), commonly known as Geraldton waxflower, is a perennial evergreen shrub endemic to Western Australia (Elliot and Jones 1984). Flower buds on pedicels are initiated in pairs in the leaf axils of axillary shoots during an annual short-day transition period (Shillo *et al*. 1984). Flowering occurs in winter-to-spring. Development of floral organs proceeds from tight buds enclosed in two shiny bracteoles to open 15-20 mm-diameter flowers with five broad petals and a cup-shaped nectar-producing hypanthium (O'Brien *et al.* 1996; Olley *et al*. 1996). Flowering stems of *C. uncinatum* are harvested as a cut flower crop in Australia and overseas when between 20 and 70% of flowers are open (Beal *et al*. 1998).

Postharvest abscission of *C. uncinatum* flowers and buds during export handling is a major problem that reduces the visual appeal and value of stems (Joyce 1993). Separation occurs at the base of the floral tube, although the precise anatomy of abscission in *C. uncinatum* has not been characterised. Ethylene, either synthesised by the floral organs in response to abiotic and biotic stresses (e.g. water deficit, pathogen infection) or arising from exogenous sources (e.g. automobile exhaust fumes) mediates abscission (Joyce 1988, 1993). Previous investigations by Joyce (1988, 1989) and Macnish *et al*. (2000) established that treatments with 7-10 μL L^{-1} ethylene for 12 to 24 h at 20°C induced abscission of open nectiferous flowers from several *C. uncinatum* cultivars, including 'Purple Pride' and 'Alba'. However, the sensitivity of floral organs at other development stages to ethylene-induced abscission has not been ascertained. In addition to botanical interest, characterisation of the morphology and anatomy of abscission zone tissues and quantification of the relative ethylene sensitivity of

different floral organ development stages could provide useful information to facilitate the development of practical commercial strategies for minimising abscission during postharvest handling and transport.

With a view to better characterising the separation process in *C. uncinatum*, the current study investigates the relative sensitivity of *C. uncinatum* floral organs at different stages of bud through flower development to ethylene-induced abscission. The morphology and anatomy of ethylene-responsive tissues at the floral organ abscission zone are described.

Materials and methods

Plant material

Flowering stems of commercial *C. uncinatum* cultivars were harvested from 5-8-year-old clonally propagated plants on farms near Gatton (27° 34' S, 152° 17' E; cvv. 'Purple Pride', 'Fortune Cookie') and Crows Nest (27° 16' S, 152° 03' E; cv. 'Paddy's Late') in south-east Queensland, Australia. 'Purple Pride', 'Fortune Cookie' and 'Paddy's Late' are early, mid- and late flowering *C. uncinatum* cultivars, respectively. Stems were harvested with 20-50 % opened flowers. The cut stem ends were immediately stood into deionised water and transported to the laboratory within 1 h of harvest.

Abscission zone morphology and anatomy

Thirty floral organs at each of the 10 stages of development described by Olley *et al.* (1996) (Fig. 1*a-j*) were excised at the base of their pedicels from harvested stems of

each cultivar. The morphology of the floral organ abscission zone was observed for three to five floral organs at each development stage with an Olympus SZH-ILLD light microscope (Olympus Optical Co., Ltd, Tokyo, Japan) and photographed using an Olympus PM-10AK photomicrographic system with Kodak 200 ISO film. The remaining detached floral organs were used for examination of abscission zone anatomy. Tissues from members of the Myrtaceae, such as *Thryptomene calycina* (Lindl.) Stapf, can be difficult to preserve by chemical fixation due to large quantities of oils and phenolics in their tissues (Beardsell *et al*. 1993). The following procedure gave relatively successful fixation, dehydration and embedding of *C. uncinatum* tissues.

Approximately 2-mm-square blocks of tissue containing the abscission zone and adjoining pedicel and floral tube cells were dissected at each floral development stage. Excised tissues were fixed for 14 h at 20°C in a solution of 3% (v/v) glutaraldehyde (electron microscopy grade; ProSciTech, Thuringowa Central, Qld, Australia) in 0.1 M potassium phosphate buffer (pH 7.2) containing 1% (w/v) anhydrous caffeine (Sigma Chemical Co., St Louis, MO, USA). The addition of caffeine to the fixative solution was to prevent phenolics in the vacuoles from leaching into the cytoplasm (Mueller and Greenwood 1978; O'Brien 1995). The fixative was infiltrated into explants under reduced pressure of 93 kPa for the first 10-20 min to improve penetration (O'Brien and McCully 1981). Fixed explants were washed in three sequential 10 min changes of the same 0.1 M phosphate buffer and caffeine solution described above. They were then dehydrated in a graded series of 5, 10, 15, 20, 25, 30, 40, 50, 60, 70, 80, 90, 100 and 100% again (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; Spurr 1969) in sequential graded concentrations of 50, 75 and 100% resin in acetone for 12 h each, followed by five 12 h changes in 100% resin. Explants were then embedded into fresh Spurr's resin and cured for 3 days at 60°C.

Longitudinal and transverse 0.5 μm-thick tissue sections through three to five replicate abscission zones for each stage were cut using a glass knife on an Ultracut E microtome (Reichert-Jung, Austria). The sections were placed on droplets of distilled water on glass 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 and ×4, ×10 and ×40 objective lenses and a ×10 eyepiece lens. The sections were photographed using the same photomicrographic system and film as used in the examination of abscission zone morphology.

The ultratructure of the abscission zone was examined using transmission electron microscopy. Abscission zone tissue explants from open flowers with a nectiferous pink hypanthium (i.e. development stage 7; Fig. 1*g*) were fixed as described above for light microscopy. They were then post-fixed in 1% (v/v) osmium tetroxide (spectroscopy grade; ProSciTech) in 0.1 M sodium cacodylate buffer (pH 7.2) (O'Brien and McCully 1981) for 2 h at 20°C. Tissues were dehydrated in a graded acetone series, and embedded in Spurr's resin as described above for light microscopy. Embedded explants were further trimmed around the abscission zone with a razor blade to give

segments that were approximately 1 mm^2. Longitudinal 90 nm-thick tissue sections through abscission zones were cut using a diamond knife (Drukker International, Cuijk, The Netherlands) on a Reichert-Jung Ultracut E microtome. Sections were floated on deionised water and expanded by exposure to chloroform (AR grade, Ajax Chemicals, Auburn, NSW, Australia) vapour to remove wrinkles. They were collected on 3 mm-diameter copper slot grids coated with pioloform (Agar Scientific Ltd, Stansted, Essex, UK). Sections were then stained for 2 min with 5% uranyl acetate in 50% methanol (Watson 1958), followed by 1 min in lead citrate (Reynolds 1963). They were viewed with a JEOL JEM1010 transmission electron microscope (JEOL Ltd, Tokyo, Japan) at 80 kV and photographed in digital form using Soft Imaging analySIS® software (Soft Imaging System GmbH, Munster, Germany).

The surface area occupied by protoplasm (i.e. cytoplasm plus nucleus) and vacuoles in single abscission zone and proximal pedicel cells was estimated in the transverse plane. Transmission electron photomicrographs of longitudinal sections through abscission zones and adjacent pedicel cells were traced onto paper. The tracings of protoplasm versus vacuoles within each protoplast were cut out and weighed separately using a four decimal-place Sartorius A120S analytical scale (Sartorius GmbH, Gottingen, Germany). Relative transverse plane surface areas of protoplasm and vacuoles were expressed as a proportion (%) of the total surface area of the protoplast. Data on the relative proportions of protoplasm and vacuole in sectioned cells are presented as means ± standard errors (n=15). The data were also arcsine transformed to approximate a normal distribution and 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 evaluate the significance of differences between means.

Sensitivity of floral organs to ethylene

'Purple Pride' stems bearing at least nine floral organs at each of three development categories (viz. young bud, expanding bud, and open flower) were trimmed to 20 cm-length. These categories corresponded to the floral development stages 1, 2 to 4, and 5 to 9, respectively (Fig. 1*a*-*i*). Leaves from the lower 10 cm of stems were removed. Stem ends were then recut under deionised water, removing 1 to 2 cm from the base to avoid air embolisms. They were stood individually into 150 mL capacity glass flasks containing 100 mL deionised water. The opening of each flask was closed around stems with Parafilm® laboratory film (American National Can™, Chicago, IL, USA) to prevent abscising flowers and leaves from contaminating the solution.

Stems in flasks (n=10) were placed individually into sealed 60 L glass chambers that were arranged in a completely randomised design within a 20°C room. Two 100-mL glass beakers both containing 10 mL of 1 M potassium hydroxide (AR grade, Ajax Chemicals) and a saturated filter paper oriented vertically were kept in each chamber to absorb CO_2 from respiration. An ethylene stock was prepared by diluting 98% (v/v) ethylene (industrial grade; BOC Gases Australia Ltd, North Ryde, NSW, Australia) in the air inside a glass volumetric flask sealed with a rubber plug. A solution of saturated ammonium sulphate (AR grade; Chem-Supply Pty Ltd, Gillman, SA, Australia) was injected into the inverted flask to provide an additional seal on the inside of the rubber plug. Aliquots of stock ethylene gas were then injected through a rubber septum in the

lid of each chamber using a syringe. Flowers were treated with either 0 (control) or 100 μL L^{-1} ethylene for 24 h. The relatively high ethylene concentration of 100 μL L^{-1} was used to saturate flowers and ensure the maximum abscission response was induced. Control stems were kept in matching glass chambers, but in addition, each held a second 100 mL glass beaker containing 10 g of Purafil® (aluminium oxide pellets impregnated with $KMnO_4$; Purafil Inc., Doraville, GA, USA) to oxidise ethylene produced by the plant material. Floral organ abscission was assessed at 12, 18 and 24 h after the commencement of treatment as the number of floral organs for each development category to separate from their pedicels. Count data were expressed as a cumulative proportion (%) of abscised floral organs relative to the initial number on a stem.

Ethylene concentrations of the stock and in-treatment chambers were quantified by gas chromatography (Taylor *et al*. 1997). A Shimadzu GC-8AIF gas chromatograph (GC) fitted with a flame ionisation detector operating at 120°C was used to measure ethylene in 1-mL air samples from the stock flask and treatment chambers. The 1.2 m-long, 3.5 mm-internal diameter glass GC column was packed with activated alumina (mesh size 80/100) and maintained at 90°C. A 0.09 or 103 μL L^{-1} ethylene gas standard (β-grade special gas mixture; BOC Gases Australia Ltd) was used to calibrate the GC.

The sensitivity of different floral organ development stages to a range of ethylene concentrations and various exposure times were also determined. In these experiments, four floral development stages (viz. stage 1, flower bud with shiny bracteoles; stage 4, bud with petals beginning to lift; stage 7, flower with nectiferous pink hypanthium;

stage 10, senescing flower with closing petals; Fig. 1*a*, *d*, *g* and *j*, respectively) were examined. 'Purple Pride' floral organs at the four stages were excised at the base of their pedicels. The pedicels were each inserted into individual 1.5 mL capacity microfuge tubes through a small hole made in the lid. The tubes contained 1.5 mL deionised water at 20°C. Floral organ explants in tubes were randomly assigned to treatments and exposed to ethylene inside chambers as described above. In the ethylene concentration experiment, floral organs were treated with 0 (control), 0.01, 0.1, 1, 10 and 100 μL L^{-1} ethylene for 12 h at 20°C. In the exposure time experiment, floral organs were treated with 1 μL L^{-1} ethylene for 0 (control), 3, 6, 9, 12 and 24 h at 20°C. In both experiments, treatments were each comprised of 50 replicate floral organs. These two experiments were also once-repeated for floral organs from separate harvests at 3-day intervals.

After treatment, floral organs in microfuge tubes were removed from the treatment chambers and immediately assessed for abscission. For floral organs that had not already abscised during treatment, light finger pressure was applied against the side of the pedicel at a position 5 mm below the abscission zone. Pressure was applied either until abscission occurred or the base of the pedicel moved to make a 45° angle to the floral tube. Finger pressure was determined using a Shimadzu AGS-H Instron (Shimadzu Corp., Kyoto, Japan) to be around 0.56 N. Abscission was expressed as the proportion (%) of floral organs in each treatment that separated either during treatment or in response to the finger pressure test.

Abscission data were presented as binary responses, either abscised (1) or not abscised (0). Data means ± standard errors are shown. Abscission data from the first sensitivity experiment were statistically modelled according to the time that abscission occurred. The number of floral organs at each of three development categories that abscised from stems during the time intervals of 0 to 12 h, 12 to 18 h, and 18 to 24 h was calculated. The number of floral organs that were retained on stems at the completion of the 24 h experiment was also recorded. Collectively, these four responses were processed as multinomial logistic regressions using the Genmod procedure of SAS Release 8.2 (SAS Institute Inc., Cary, NC, USA) (Dobson 2002). The binary abscission response data from the duplicated experiments 2 (i.e. concentration) and 3 (i.e. exposure time) were pooled (n=100) for presentation and processed as logistic regressions using the Logistic procedure of SAS. The regressions described relationships between floral development, treatment variables (i.e. time, concentration) and abscission (Dobson 2002). Treatments in which no floral organs abscised were excluded from the analysis. Wald chi-square tests (P=0.05) were used to evaluate the effects of development stage and ethylene treatments on abscission responses.

Anatomy of ethylene-induced abscission

Relationships between the sensitivity of 'Purple Pride' floral organs to ethylene-induced abscission and the anatomy of abscission zone tissues were studied using scanning and transmission electron microscopy and light microscopy. Stage 1 and 7 floral organs were excised at the base of their pedicels from stems. Floral organs of *C. uncinatum* are borne as pairs and develop synchronously (Shillo *et al*. 1984; O'Brien *et al*. 1996). For light and transmission electron microscopy, floral organs were removed

from stems as replicate pairs for ethylene treatments. They were inserted into microfuge tubes containing deionised water and treated with either 0 (control) or 10 μL L^{-1} ethylene for 12 and 24 h at 20°C inside glass chambers as described above for ethylene sensitivity experiments. Eighty replicate floral organs at each stage of development were used for each treatment.

Following ethylene treatment, twenty replicate floral organs at each development stage and each ethylene treatment were selected at random. Approximately 2-3-mm-square blocks of pedicels and floral tube tissues containing the abscission zones were then dissected out. Abscission zone explants were excised from stage 1 and 7 floral organs that either abscised during ethylene treatment or subsequently separated when finger pressure was applied to the pedicel as described above. Additional explants were cut from treated and control (i.e. not ethylene-treated) floral organs that remained attached to their pedicels when finger pressure was applied to the pedicel. Explants were also cut from control floral organs not subjected to the finger pressure test. For scanning electron microscopy, explants were excised from control floral organs forcibly separated at the abscission zone by finger pressure.

Scanning electron microscopy was used to examine the morphology of cell separation at the floral organ abscission zone. Explants were chemically fixed as described above for transmission electron microscopy. They were then rinsed for 10 min in deionised water, and dehydrated in sequential graded concentrations of 30, 40, 50, 60, 70, 80, 90 and 100% (v/v) ethanol for 10 min at each step. In order to fully dehydrate the tissues, explants were immersed five more times in fresh 100% ethanol for 10 min each.

Dehydrated explants were critical point dried by substituting the ethanol with liquid CO_2 (O'Brien and McCully 1981). Explants in ethanol were infiltrated with food grade CO_2 gas (BOC Gases Australia Ltd) at 13°C and 5515 kPa for 15 min inside a Polaron E3000 critical point drying apparatus (Polaron, Watford, Hertfordshire, UK). The apparatus was then vented for 2 min to release the ethanol solvent. The infiltration and venting process was repeated three times until the CO_2 replaced all the ethanol in explant tissues. The temperature and pressure inside the apparatus were increased to 35°C and 8273 kPa, respectively, to exceed the critical drying point of CO_2. The dried explants were withdrawn from the apparatus and mounted on aluminium stubs using double-sided adhesive tape. They were then enclosed in a coating unit (SPI Supplies, West Chester, PA, USA) and sputter coated with gold. Five to six replicate explants were viewed with a JEOL JSF6300 field emission scanning electron microscope at 5 kV and photographed in digital form by means of Image Slave software (OED Pty Ltd, Hornsby, NSW, Australia).

Remaining abscission zone explants were processed for light and transmission electron microscopy as described above for the study of abscission zone morphology. For light microscopy, longitudinal tissue sections through three to five replicate abscission zones were cut to 0.5-μm-thick using a glass knife as described above. The sections were stained with toluidine blue O as described above. Stained sections were viewed under bright field illumination with an Olympus BH-2 light microscope and photographed with the photomicrographic system and film used to study abscission zone morphology. For transmission electron microscopy, longitudinal tissue sections through three to five replicate abscission zones were cut to 90-nm-thick using a diamond knife as described

above. The sections were collected on copper slot grids coated with pioloform, stained with 5% uranyl acetate in 50% methanol, followed by treatment with lead citrate for 1 min and viewed with a JEOL JEM1010 transmission electron microscope at 80 kV as outlined above.

The integrity of vascular tissues across the floral organ abscission zone during ethylene-mediated separation was examined using a tracer dye and fluorescence microscopy. In this study, flowering 'Purple Pride' stems were trimmed to 10 cm-length sprigs as outlined for the first ethylene sensitivity experiment. The cut stem ends were stood into 1.5 mL microfuge tubes containing 1 mL deionised water. Sprigs in tubes (n=10) were placed into glass chambers (n=2) and treated with 0 (control) and 10 μL L^{-1} ethylene for 12 h at 20°C as described above. At the completion of treatment, lids to chambers were carefully removed. Sprigs were then pulsed with either 0.1% (w/v) disodium fluorescein (uranin) (Sigma Chemical Co.) in deionised water (Polito 1999) or deionised water (control) alone for 30 min at 20°C.

For stage 7 flowers that abscised from sprigs during the uranin pulse treatment, approximately 3 mm-long segments of either the floral tube or pedicel that included the abscission zone were excised. Free-hand longitudinal sections about 0.5 mm-thick were cut through the central axis of these explants using a stainless steel razor blade. Sections were placed into droplets of deionised water on microscope slides. They were viewed under ultra violet (UV) illumination (HBO 100) using an Olympus BX60 light microscope with the fluorescence filter set U-MWIBA, which allowed excitation with 460-490 nm light and restricted emission to > 505 nm. Sections were photographed in

digital form using Cool SNAP imaging software (Roper Scientific Inc., Tucson, AZ, USA). A stage micrometer was also photographed and used to determine the scale on all light photomicrographs.

Results and discussion

Abscission zone morphology and anatomy

The floral organ abscission zone in *C. uncinatum* cv. 'Purple Pride' is a morphologically and anatomically distinct band of tissues that forms at the pedicel-floral tube junction during floral development (Fig. 1*k-n*). The morphological features are similar for cultivars 'Fortune Cookie' and 'Paddy's Late'. The zone is initially evident as a narrow constriction of tissue at the base of stage 1 and 2 flower buds (fb) that is partially obscured from external view by two overlapping bracteoles that fuse to the pedicel (p) (Fig. 2*a*). Similarly, bracteoles were observed to obscure the abscission zone in *Phaseolus vulgaris* and in oil palm (*Elaeis guineensis* Jacq.) reproductive organs (Webster and Chiu 1975; Henderson and Osborne 1994). Once bracteoles are shed and throughout subsequent development of the floral organ (i.e. stages 3-10; Fig. 1*c-j*), the abscission zone in *C. uncinatum* is a distinct indentation at the junction of the floral tube (ft) and pedicel (Fig. 2*b*). The general anatomy of the abscission zone junction in *C. uncinatum* cultivars 'Purple Pride', 'Fortune Cookie' and 'Paddy's Late' was similar for sequential stages of floral development following detachment of bracteoles from the floral tube (images not shown). These abscission zone features are similar to those described for other species that normally shed floral organs, such as bean (*Phaseolus vulgaris* L.), *Hibiscus rosa-sinensis* L., and tomato (*Lycopersicon esculentum* L.) (Webster and Chiu 1975; Gilliland *et al*. 1976; Roberts *et al*. 1984).

In longitudinal tissue sections, the abscission zone was comprised of a narrow file of five to six rows deep of small, flattened and closely packed parenchyma cells at the pedicel-floral tube junction (Fig. 2*c*). The zone is readily distinguished from the larger isodiametric (symmetrical) parenchyma cells of the distal floral tube and the large laterally flattened and loosely packed cells of the proximal pedicel. Similarly, the floral organ abscission zones in *Phaseolus vulgaris*, *Hibiscus rosa-sinensis* and *Lycopersicon esculentum*, have been characterised as a compact band of three to ten rows of small flattened or isodiametric cells (Webster and Chiu 1975; Gilliland *et al*. 1976; Roberts *et al*. 1984).

In transverse sections, the abscission zone in *C. uncinatum* was confined to a ring of cells four to five rows wide that formed inside the epidermis and ended *c*. 150 μm from the central vascular tissues (v) (Fig. 2*d*). Inside the band of flattened abscission zone cells were cell divisions (cd) and rounded cells with large intercellular spaces (is). Cells both distal and proximal to the abscission zone and adjacent to the central vascular tissues in *C. uncinatum* contained inclusions of irregular morphology (e.g. Fig. 2*d*). The distribution and morphology of the inclusions were similar to those identified as aggregate calcium oxalate crystals in parenchyma cells of *C. uncinatum* cvv. 'Purple Pride', 'Fortune Cookie' and 'Paddy's Late' (Macnish *et al*. 2003). In other species including *Phaseolus vulgaris*, *Hibiscus rosa-sinensis*, and *Phyllanthus niruri* L., calcium oxalate crystals have been observed in the cells of the floral organ abscission zone region prior to cell separation (Webster and Chiu 1975; Gilliland *et al*. 1976; Grimson and Arnott 1983). Following abscission of the organ, the crystals at the

proximal separation cell layer may protect tissues against invading insects and pathogens (Grimson and Arnott 1983).

Abscission zones are also generally distinguished as a band of highly protoplasmic cells that persistently divide and fail to enlarge and vacuolate along with surrounding tissues (Sexton and Roberts 1982; Bleecker and Patterson 1997). These cellular features were also evident in *C. uncinatum*. Abscission zone cells had a greater proportion of protoplasm (e.g. 30.5 ± 3.5%, n=15) and correspondingly less vacuole (e.g. 69.5 ± 3.5%, n=15), in terms of longitudinal plane surface area in section, than highly vacuolated (e.g. 96.0 ± 0.5%, n=15) neighbouring pedicel parenchyma cells. The presence of mitochondria (m) and chloroplasts (ch) in the cytoplasm of floral organ abscission zone cells of *C. uncinatum* (Fig. 2*e*) suggests that these cells are metabolically active. Fragments of endoplasmic reticulum (er) were observed in some sections. The abscission zone also had broad areas of middle lamella between the walls of neighbouring cells (images not shown). Cell divisions in *C. uncinatum* at the distal floral tube end of the abscission zone (images not shown) are consistent with similar observations in *Hibiscus rosa-sinensis*, soybean (*Glycine max* Merrill.) and *Pelargonium* × *hortorum* Bailey floral organ abscission zone cells (Gilliland *et al*. 1976; Oberholster *et al*. 1991; Evensen *et al*. 1993).

The abscission zone cells at the intersection of the pedicel and floral tube in stage 1 and 2 buds enclosed by bracteole tissue were similar in size and shape to those described for floral organs harvested at subsequent stages of development (images not shown). However, a layer of four to five small flattened cells with thin walls delineated the

junction between each bracteole and the pedicel (Fig. 2*f*). This cell layer merged with the laterally elongated cells that lined the outer, upper surface of pedicels and may function as a separation layer for the shedding of bracteoles during abscission of enclosed buds. Separate abscission zones for different tissues (e.g. tepals, bracteoles) that comprise reproductive organs have also been reported in *Elaeis guineensis* (Henderson and Osborne 1994). These observations may have implications for the progress of abscission in *C. uncinatum* floral buds compared with open flowers. For instance, if cells at one abscission zone separate, and the cells at the other zone remain intact, the bud may not readily abscise.

Sensitivity of floral organs to ethylene

Treatment of *C. uncinatum* cv. 'Purple Pride' flowering stems with 100 μL L^{-1} ethylene for 12 to 24 h at 20°C induced abscission of floral organs from pedicels at the abscission zone (e.g. Fig. 3*a*). The level of abscission varied among floral organs at different stages of development. Expanding buds (i.e. stages 2-4) and open flowers (i.e. stages 5-9) were generally more sensitive to treatments with 100 μL L^{-1} ethylene than young buds with shiny bracteoles (i.e. stage 1) (Fig. 4). Ethylene-treated stage 1 buds often abscised at the floral tube base, but remained attached to the pedicel by the bracteole tissues (Fig. 3*b*). These observations could have implications for the cut flower trade, wherein stems with floral organs at particular stages of development are used (Beal *et al*. 1998). For example, stems predominately bearing immature stage 1 flower buds may be less responsive to ethylene-induced abscission than stems bearing more mature buds and flowers. Similarly, with *C. uncinatum* floral organ explants, stage 1 flower buds enclosed in shiny bracteoles were relatively less sensitive (i.e. 49%

abscission) than stage 4 buds (i.e. 88% abscission) to treatment with 1 μL L^{-1} ethylene for 12 h at 20°C (Fig. 5*a*). However, both stage 1 and 4 buds were similarly responsive to treatment with either 10 or 100 μL L^{-1} ethylene. In general, abscission of floral organ explants in response to treatments with 1 μL L^{-1} ethylene for 12 and 24 h at 20°C was greatest for stage 4 buds and stage 7 nectiferous flowers and least for stage 10 aged (senescent) flowers (Fig. 5*a*, *b*).

A similar decrease in sensitivity as found for *C. uncinatum* flowers to ethylene with development was also reported for several *Dianthus caryophyllus* L. lines (Mayak and Tirosh 1993; Onozaki *et al*. 2001). Sensitivity of plant tissue to ethylene may depend on the concentration and/or affinity of ethylene receptors and the capacity for downstream metabolism (Tieman and Klee 1999), and both may be lower at advanced stages of development. Alternatively, the apparent loss in responsiveness of senescent stage 10 flowers may be due to development of lignified secondary cell wall thickening in the xylem vessels that traverse the abscission zone. These thickenings may increase the resistance of the xylem vessels to fracturing (Sexton and Roberts 1982) and the flowers may be reluctant to abscise.

Treatment of *C. uncinatum* cv. 'Purple Pride' floral organs with ethylene at low concentrations (e.g. 0.1-1 μL L^{-1}) and for short exposure periods (e.g. 6-9 h) at 20°C was sufficient to initiate abscission (Fig. 5*a*, *b*). Increasing the ethylene concentration and/or treatment period generally increased the level of floral organ abscission. There was no abscission of control floral organs kept in 0 μL L^{-1} ethylene. These results were similar to those reported by Joyce (1988), where 7 μL L^{-1} ethylene applied for 4 h at

20°C initiated flower abscission from *C. uncinatum*. The relatively rapid abscission response of *C. uncinatum* cv. 'Purple Pride' floral organs to ethylene is consistent with floral organ abscission from other ornamental species (e.g. *Geranium robertianum*, *Pelargonium* × *hortorum*; Sexton *et al*. 1983, 1985; Evensen *et al*. 1993). This rapid response to ethylene presumably reflects the capacity of abscission zone cells to readily initiate changes at biochemical (e.g. secretion of cell wall hydrolases) and anatomical (e.g. rounding up of separation layer cells) levels that cause cell separation (Sexton and Roberts 1982; Evensen *et al*. 1993).

Anatomy of ethylene-induced abscission

Treatment of stage 1 flower buds with 10 μL L^{-1} ethylene for 24 h at 20°C induced their separation from pedicels at both a central abscission zone and at the peripheral edges where bracteoles joined to the pedicels. Examination of abscised surfaces on the pedicel (Fig. 6*a*) and floral tube (Fig. 6*b*) by scanning electron microscopy showed that the central abscission zone was comprised of a ring of intact, apparently turgid, rounded cells that encircled the central vascular tissues. The central vascular tissues (v) that lead into the floral tube from the pedicel tore apart during abscission leaving a stump on the pedicel surface (Fig. 6*c*) and matching cavities in the floral tube (images not shown). The epidermal cells at the abscission zone margins were largely round and intact (images not shown). These intact and rounded cells also surrounded torn vascular strands (v) at the base of bracteoles (br) that enclosed separated floral tubes, and appear to constitute a separate abscission zone (Fig. 6*d*).

Light microscopy confirmed that bracteoles separated at their base from the side of the pedicel at a separate abscission zone. The five to six rows of small, laterally elongated cells that comprised the abscission zone generally separated as intact and apparently turgid cells (Fig. 6*e*). Thus, in stage 1 buds, abscission proceeds via separation at separate abscission zones for the floral tube and the bracteoles. In oil palm (*Elaeis guineensis*), individual abscission zones also exist for the fruit and adjacent tepals and rudimentary androecium tissues (Henderson and Osborne 1994).

The secondary bracteole abscission zone in *C. uncinatum* appeared to be less responsive to ethylene than the primary central abscission zone at the junction of the pedicel and floral tube. For example, treatment with 10 μL L^{-1} ethylene for 12 h at 20°C induced the partial separation of some abscission zone cells at the base of the floral tube, but did not cause separation of cells at the base of bracteoles (images not shown). Rather, these cells tore unevenly from the upper surface of the pedicel when finger pressure was applied. Increasing the ethylene exposure period from 12-24 h was required to elicit even separation of bracteoles at their abscission zone. Similarly, in *Elaeis guineensis*, abscission of fruit that were enclosed by rudimentary androecium tissues was bi-phasic (Henderson and Osborne 1994). Differences in the ethylene responsiveness of *C. uncinatum* bracteole and floral tube abscission zones may lie in their differentiation as vegetative and reproductive tissues, respectively. Relative to vegetative organs, ethylene-induced abscission of floral organs is typically rapid (e.g. in 1-12 h versus 24-48 h) (Sexton *et al*. 1985).

Treatment of stage 7 flowers with 10 μL L^{-1} ethylene for 12 h at 20°C induced abscission upon application of light finger pressure to the pedicel. When viewed with the scanning electron microscope, the abscised surfaces of floral tubes and their pedicels (images not shown) generally displayed similar features to those described above for ethylene-treated flower buds. In contrast to ethylene-treated buds and flowers, the forcible removable of control (i.e. no ethylene) floral tubes from their pedicels caused an uneven tearing of all tissues above the abscission zone, leaving a fractured stump of the floral tube attached to the pedicel (images not shown). In control stage 1 buds, tissues at the base of each bracteole also tore extensively during forced separation of floral tubes (images not shown).

Ethylene-induced abscission of *C. uncinatum* flowers and buds occurred at a narrow two to four cell wide separation layer at the distal end of the abscission zone. The separation layer cells on the abscised surface of both the pedicels and floral tubes were typically spherical and loosely packed (Fig. 6*f*, *g*). In line with observations for other species (e.g. *Phaseolus vulgaris* L., *Geranium robertianum* L., *Pelargonium* × *hortorum* Bailey), abscission of *C. uncinatum* floral organs involved extensive degradation of the middle lamella from around the abscission zone cell walls (Fig. 6*h*) (Webster and Chiu 1975; Sexton *et al*. 1983; Evensen *et al*. 1993). Breakdown of the pectin-cementing middle lamella coupled with the apparent maintenance of turgor in separation layer cells probably accounts for the expansion of these cells as is typically seen in leaf and floral organ abscission zones (Sexton and Redshaw 1981; Sexton *et al*. 1983). The resulting pressure exerted on adjacent cells causes separation along the plane of the degraded middle lamella (Sexton and Redshaw 1981; Evensen *et al*. 1993).

The smooth surfaces of abscised *C. uncinatum* separation layer cells (Fig. 6*a*, *b*) was similar to observations made by Joyce and Beal (1999) on *Grevillea* 'Sylvia' flower tepal separation surfaces. Through contrasting the rough cell wall surfaces of physically separated tepals with the smooth surfaces of tepals separated as a consequence of normal physiological function, the authors argued that separation in *Grevillea* 'Sylvia' involved cell wall hydrolase activity. The key biochemical processes in abscission are typically enzyme-mediated dissolution of pectin in the middle lamella and cellulose in the primary wall of abscission zone cells (Sexton and Roberts 1982; Roberts *et al*. 2002). Anatomical evidence presented herein also suggests that cell wall degrading enzymes are involved in ethylene-induced floral organ abscission in *C. uncinatum.* However, measurement of abscission zone cell wall degrading enzyme activity during ethylene-induced abscission is required to support this proposal.

Following ethylene-induced abscission, the protoplasm (pr) in *C. uncinatum* separation layer cells on the exposed pedicel surface degenerated to a strand that lacked recognisable organelles (Fig. 6*h*). The protoplasm and organelles (i.e. mitochondria, nucleus) in cells located three to five layers from the separation surface were also partly degraded (Fig. 6*h*). Similarly, the protoplasm in separation layer cells of other species (e.g. *Geranium robertianum, Pelargonium* × *hortorum*) degenerates after abscission (Sexton *et al*. 1983; Evensen *et al.* 1993). In *C. uncinatum*, this protoplasm leaked out through small breaks in the cell walls to cover the external walls of cells at the separation surface (Fig. 6*h*). Degraded and exuded protoplasm may form a protective

layer against pathogen infection and desiccation (Sexton and Roberts 1982; Evensen *et al*. 1993).

Ethylene-mediated cell separation in *C. uncinatum* initially occurred in the cell layers between the central vascular tissues (v) and the peripheral epidermis (ep) (Fig. 7*a*). This finding is typical of leaf and floral organ abscission, where the epidermis and xylem vessels are the last tissues to separate. Cell separation within this region may either proceed inward from the epidermis to the vascular tissues (Gilliland *et al*. 1976), or it may start near the vascular tissues and end at the epidermis (Szymkowiak and Irish 1999). The epidermal tissues in the *C. uncinatum* abscission zone eventually separated as intact cells (images not shown), again suggesting the involvement of cell wall degrading enzymes (Sexton 1976). The route of vascular tissues in the pedicel and floral tube was visualised by pulsing a control (i.e. 0 μL L^{-1} ethylene) flower with disodium fluorescein (Fig. 7*b*). The translocation of disodium fluorescein in the vascular tissues across the abscission zone of ethylene-treated flowers just prior to their separation confirmed that the pedicel and floral tube remained attached at connecting vascular strands (Fig. 7*c* and *d*). As is commonly reported for leaf, fruit and floral organ abscission (Sexton and Roberts 1982), the final stage in the abscission of *C. uncinatum* floral organs involved the fracturing of the central vascular tissues. The vascular tissues apparently tore apart at the abscission zone in response to the mechanical stress generated by the expanding separation layer cells (Sexton and Redshaw 1981). Wind and gravity can also facilitate the tearing of these tissues (Sexton and Roberts 1982).

In conclusion, this study has shown that the morphology and anatomy of the floral organ abscission zone in *C. uncinatum* is generally similar to other species that actively shed their floral organs. As evidenced by smooth separation layer cell surfaces after abscission, ethylene-induced cell separation involved degradation of the middle lamella at the separation layer, suggesting that abscission may proceed via the activity of cell wall-degrading enzymes. Flower buds with opening petals and flowers with a nectiferous hypanthium were generally more sensitive to ethylene-induced abscission than immature flower buds enclosed in bracteoles and aged senescent flowers. Bracteoles that enclosed flower buds abscised at a separate abscission zone to that of the central floral tube. The bracteole abscission zone was also less responsive to ethylene than the central floral tube abscission zone. Thus, in a temporal sense, bracteoles reduced levels of complete bud abscission as compared to buds and flowers in which the bracteoles had been shed.

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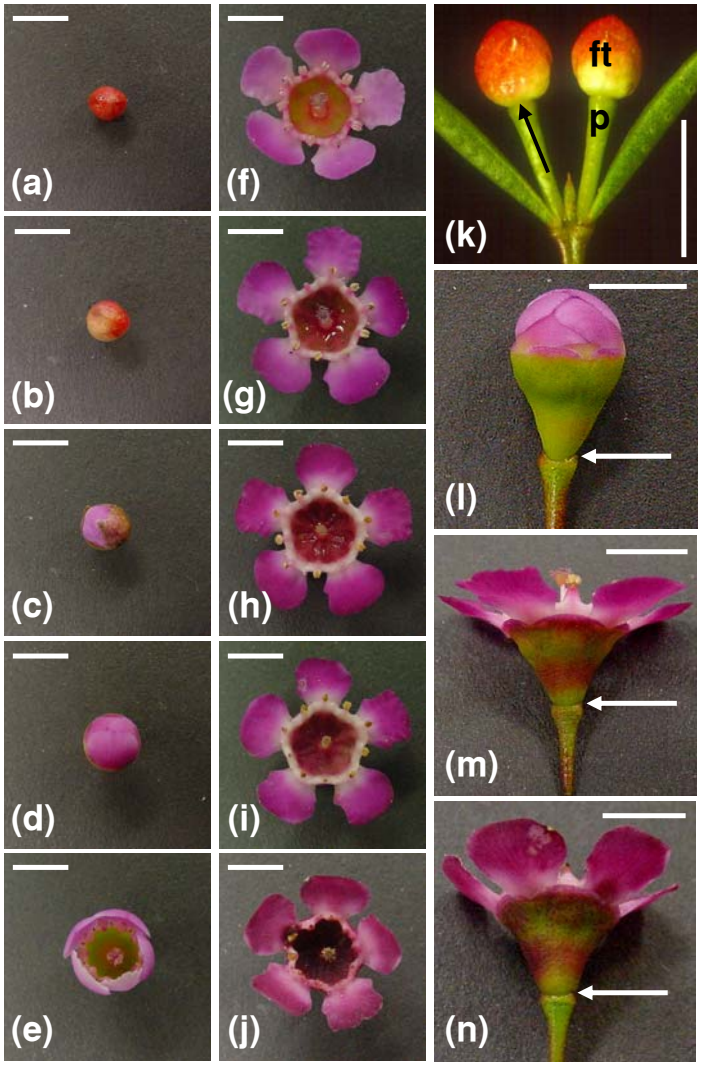
(a)
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Fig. 1. Photographs in plan and side view of the morphology of floral development and the floral organ abscission zone in *Chamelaucium uncinatum* cv. 'Purple Pride'. (*a-j*) Ten sequential stages of floral organ development; stage 1: bud enclosed in shiny bracteoles, stage 2: bud with dull senescing bracteoles, stage 3: bud with separating bracteoles, stage 4: bud with petals beginning to lift, stage 5: bud with opening petals, stage 6: flower with a nectiferous green hypanthium, stage 7: flower with nectiferous pink hypanthium, stage 8: flower with drying red hypanthium, stage 9: flower with dry red hypanthium, stage 10: senescing flower with closing petals (adapted from Olley *et al.* 1996). (*k-n*) Floral organ abscission zones of stage 1 bud with shiny bracteoles, stage 4 bud with petals beginning to lift, stage 7 flower with nectiferous pink hypanthium, and stage 10 aged (senescing flower), respectively. The arrows point to the abscission zone at the junction between the floral tube (ft) and pedicel (p). The scale bars represent 5 mm.

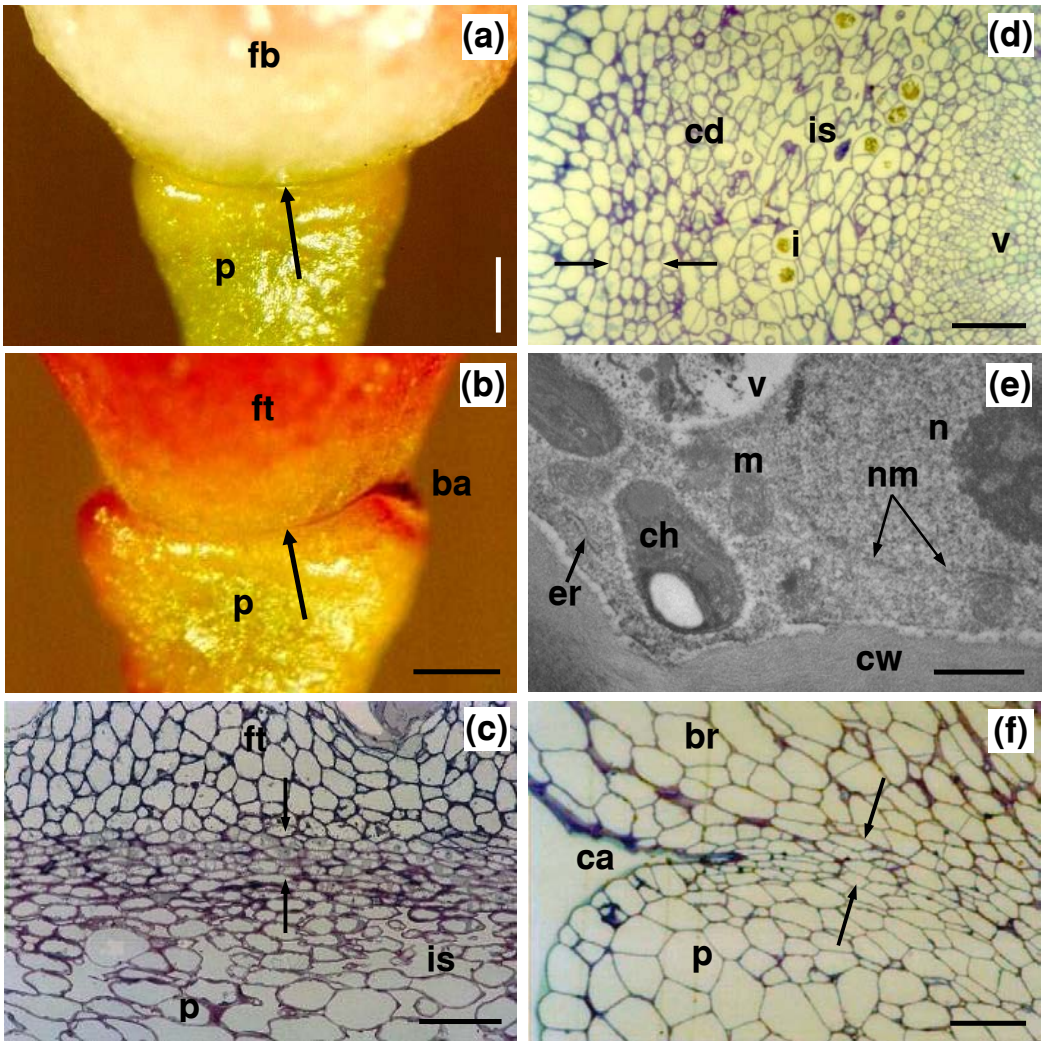

Fig. 2. Photographs of the morphology and anatomy of floral organ abscission zone tissues in *Chamelaucium uncinatum* cv. 'Purple Pride' flower buds enclosed in shiny bracteoles and flowers with a nectiferous pink hypanthium. (*a* and *b*) Side view of the abscission zone junction of a flower bud and a flower, respectively. (*c* and *d*) Light photomicrographs of a longitudinal and transverse section, respectively, through the floral organ abscission zone and adjacent pedicel and floral tube tissues of a flower. (*e*) Transmission electron photomicrograph of a longitudinal section through a typical abscission zone cell of a flower. (*f*) Light photomicrograph section through pedicel and

bracteole tissues of floral buds showing an abscission zone. The arrows in (*a*), (*b*), (*c*) and (*d*) point to the abscission zone at the junction between the floral tube and pedicel. The arrow in (*f*) points to the abscission zone at the junction between the bracteoles and pedicel. Sections in (*c*), (*d*) and (*f*) were cut to 0.5 μm-thick and stained with toluidine blue O. The section in (*e*) was cut to 90 nm-thick and stained with uranyl acetate and lead citrate. The scale bars represent 0.5 mm in (*a*) and (*b*), 50 μm in (*c*), (*d*) and (*f*) and 1 μm in (*e*). ba = bracteole attachment point, br = bracteole, ca = cavity, cd = cell division, ch = chloroplast, cw = cell wall, er = endoplasmic reticulum, fb = flower bud, ft = floral tube, i = inclusion, is = intercellular space, m = mitochondria, n = nucleus, nm = nuclear membrane, p = pedicel, pr = protoplasm, v = vacuole.

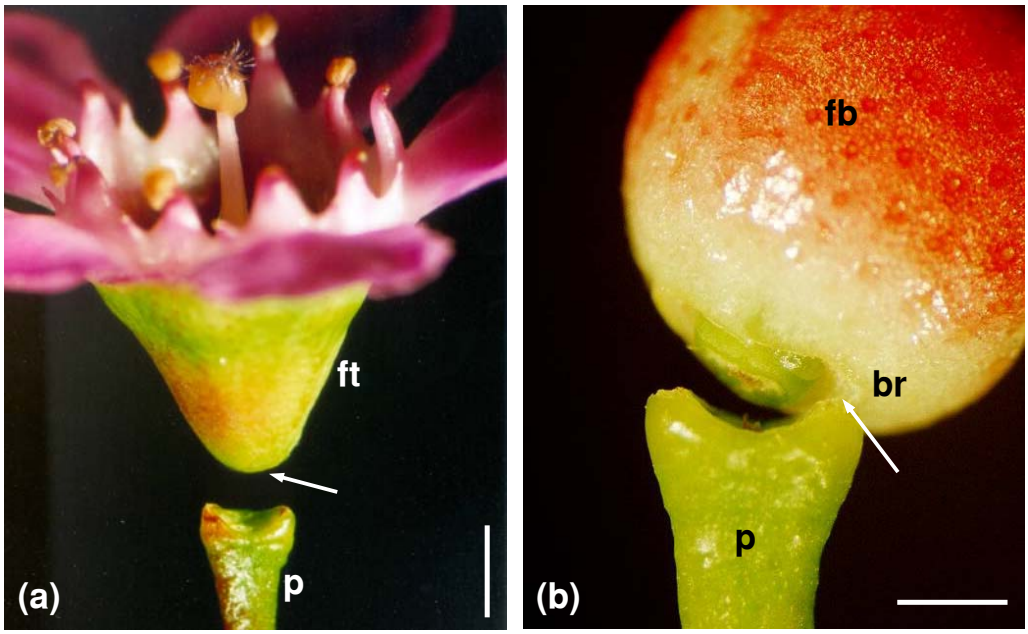

Fig. 3. Floral organ abscission responses in *Chamelaucium uncinatum* cv. 'Purple Pride'. (*a*) Separation of a flower with a nectiferous pink hypanthium from the pedicel at the abscission zone. (*b*) Partial separation of a flower bud enclosed in shiny bracteoles from the pedicel at the abscission zone. The arrow in (*a*) points to the abscission zone. The arrow in (*b*) points to bracteole tissues that remain attached to the pedicel at the abscission zone. The scale bars represent 2 mm in (*a*) and 1 mm in (*b*). br = bracteole tissue, fb = flower bud, ft = floral tube, p = pedicel.

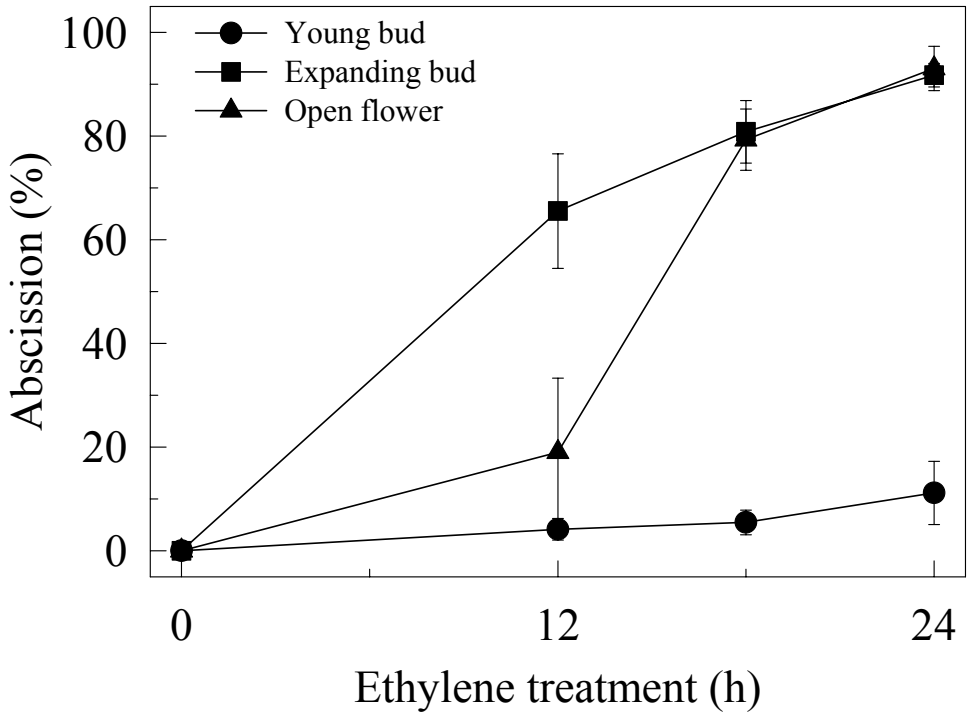

Fig. 4. Cumulative abscission (%) of *Chamelaucium uncinatum* cv. 'Purple Pride' floral organs harvested at three development categories (viz. young bud, expanding bud, open flower) to treatment with 100 μL L^{-1} ethylene for 0, 12, 18 and 24 h at 20 °C. Vertical bars show the standard errors of means (n = 5 stems). Where no vertical bars are evident, the standard error was smaller than the size of the symbol. No abscission was observed in parallel sets of control floral organs (0 μL L^{-1} ethylene). Level of significance by chi-square tests at P = 0.05 for the development category = ***. Relative differences in the abscission response between development categories based on log odds ratios (from the logistic regression analysis) were young bud versus expanding bud = ***, young bud versus open flower = ***, expanding bud versus open flower = ***. *** represents significance at $P < 0.001$.

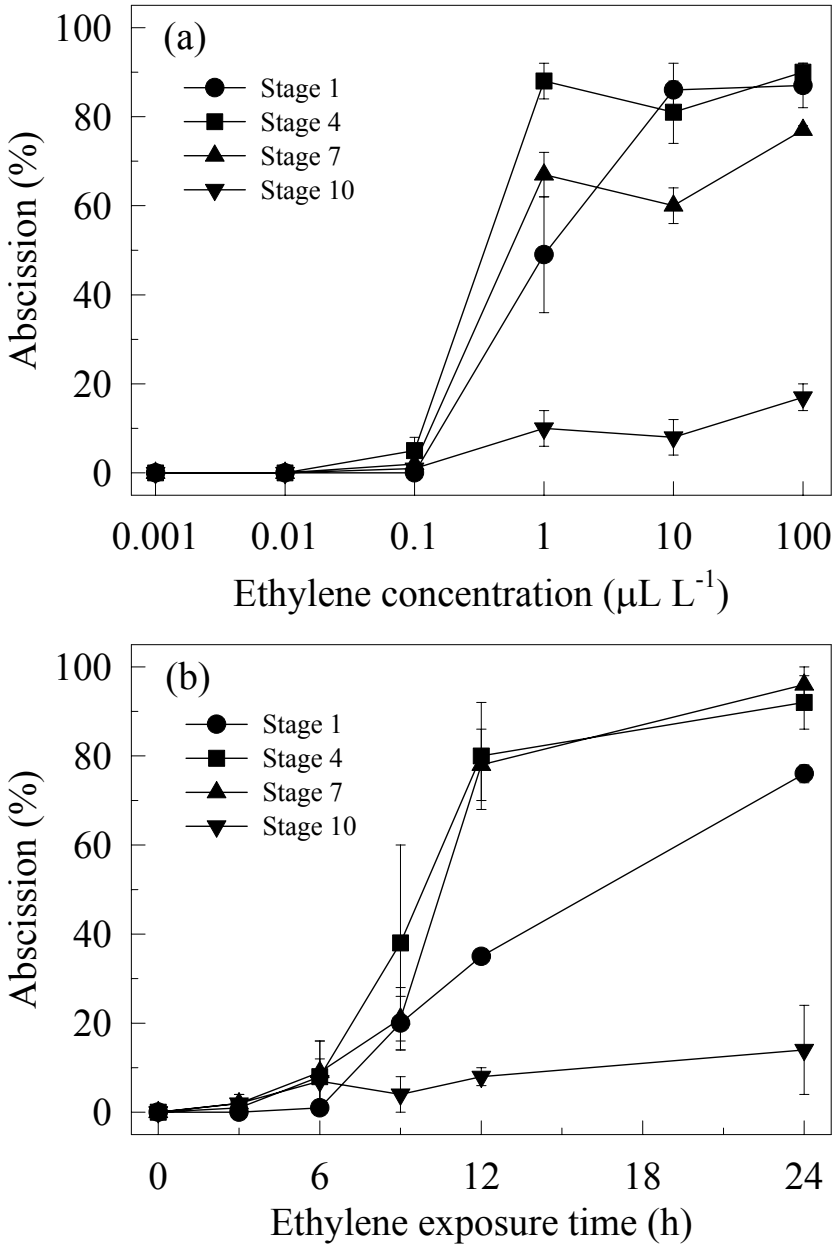

Fig. 5. Abscission responses (%) of *Chamelaucium uncinatum* cv. 'Purple Pride' floral organs to treatments with either (*a*) 0, 0.01, 0.1, 1, 10 and 100 μL L^{-1} ethylene for 12 h at 20°C or (*b*) 1 μL L^{-1} ethylene for 0, 3, 6, 9, 12 or 24 h at 20°C. Floral organs were harvested and treated at four development stages (viz. stage 1; flower bud with shiny bracteoles, stage 4; bud with petals beginning to lift, stage 7; flower with nectiferous

pink hypanthium, stage 10; senescing flower with closing petals). Data points are the mean of two duplicate experiments. Vertical bars show the standard errors of means (n=100). Where no vertical bars are evident, the standard error was smaller than the size of the symbol. No abscission was observed in parallel sets of control floral organs (0 μL L^{-1} ethylene). Levels of significance by chi-square tests at P=0.05 for treatment variables were development stage = **, ethylene concentration = ***, and stage × concentration = *** in (*a*) and development stage = ***, ethylene time = ***, and stage × treatment time = *** in (*b*). Data from treatments with 0 and 0.01 μL L^{-1} ethylene were excluded from the statistical analysis. **, and *** represent significance at P=0.01 and 0.001, respectively.

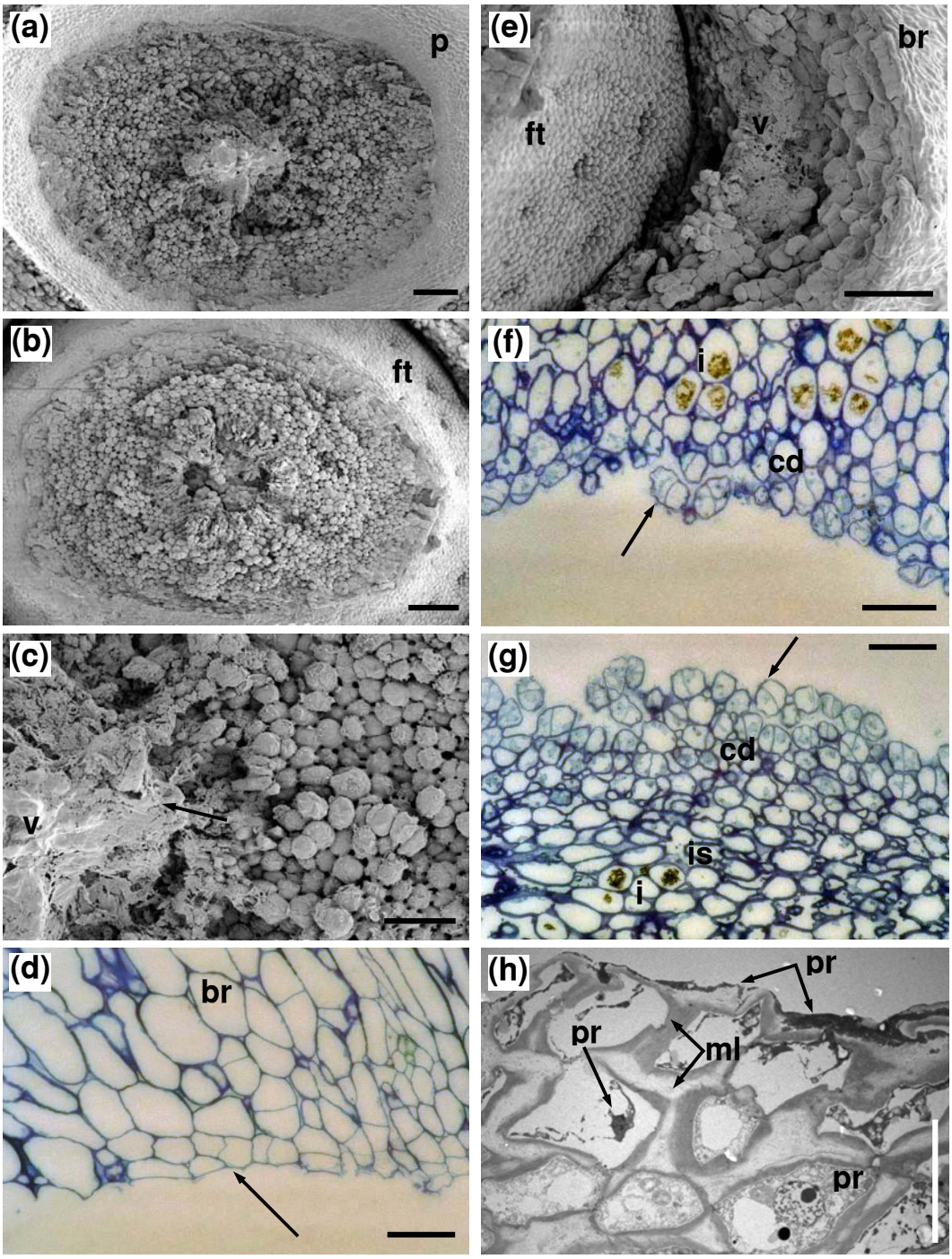

Fig. 6. Photomicrographs of abscission zone tissues of *Chamelaucium uncinatum* cv. 'Purple Pride' flower buds enclosed in shiny bracteoles and flowers with a nectiferous pink hypanthium after treatment with 10 µL L^{-1} ethylene for 24 h at 20°C. (*a*, *b* and *c*)

Scanning electron photomicrographs of flower buds showing the abscission zone tissues on the upper surface of matching pedicels and the base of abscised floral tubes. (*d* and *e*) Scanning electron and light photomicrographs of the base of a bracteole and a longitudinal section through the separation surface on a bracteole, respectively, following ethylene-induced abscission of the flower bud. (*f* and *g*) Light photomicrographs of longitudinal sections through the separation surface on floral tubes and pedicels, respectively, following ethylene-induced abscission of a flower. (*h*) Transmission electron photomicrograph of a longitudinal section through pedicel cells on the proximal side of the abscission zone of an ethylene-treated flower after separation of the floral tube. The arrow in (*c*) points to the stump of torn vascular tissues on the upper surface of the pedicel. The arrow in (*e*) points to separation of bracteole cells. The arrows in (*f*) and (*g*) point to cells at the separation surface on floral tubes and pedicels. The arrows in (*h*) point to degraded protoplasm in cells and lining the external cell wall and to degraded middle lamella. The sections in (*e*), (*f*) and (*g*) were cut to 0.5 μm-thick and stained with toluidine blue O, while the section in (*h*) was cut to 90 nm-thick and stained with uranyl acetate and lead citrate. The scale bars represent 200 μm in (*a*) and (*b*), 50 μm in (*c*), (*e*), (*f*) and (*g*), 100 μm in (*d*) and 10 μm in (*h*). br = bracteole, cd = cell divisions, ft = floral tube, i = inclusion, is = intercellular space, ml = middle lamella, p = pedicel, pr = protoplasm, v = vascular tissues.

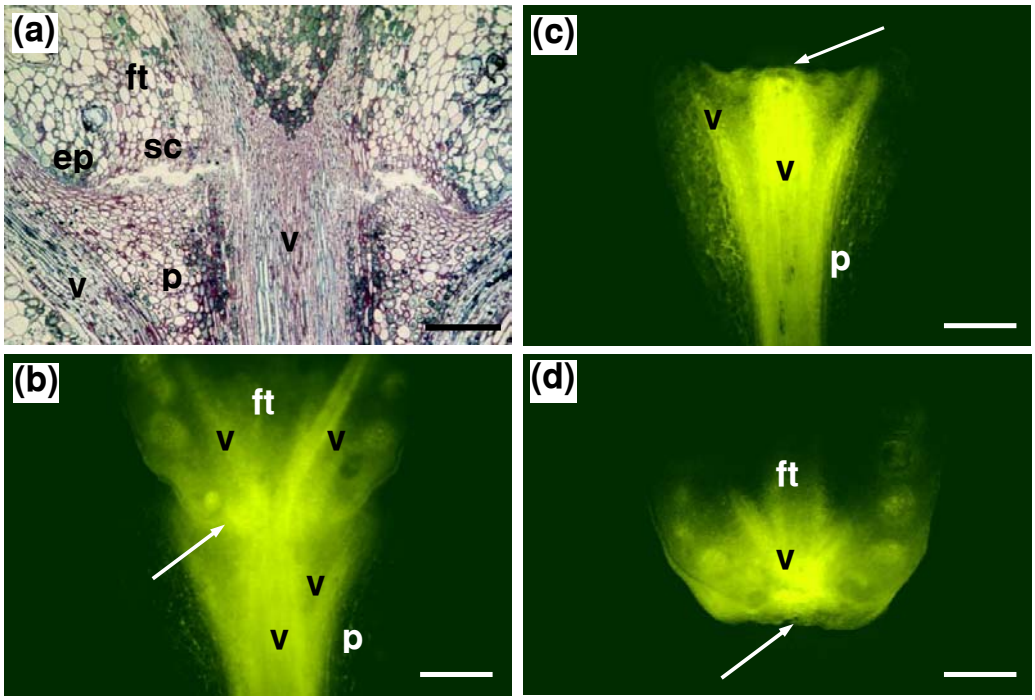

Fig. 7. Photomicrographs of floral tube and pedicel tissues of *Chamelaucium uncinatum* cv. 'Purple Pride' flowers with a nectiferous pink hypanthium after treatment with either 0 or 10 μL L^{-1} ethylene for 12 h at 20°C. (*a*) Light photomicrograph of a longitudinal section through the floral tube and pedicel tissues of a flower showing ethylene-induced separation of abscission zone cells. (*b*) Light photomicrograph of a longitudinal section through a control (i.e. 0 μL L^{-1} ethylene) flower showing the route of vascular tissues. (*c* and *d*) Light photomicrographs of longitudinal sections through a matching pedicel and floral tube following abscission induced by ethylene, respectively, showing the route of vascular tissues. The arrows in (*b*), (*c*) and (*d*) point to the abscission zone junction. The section in (*a*) was cut to 0.5 μm-thick, stained with toluidine blue O and viewed under bright field light. The sections in (*b*), (*c*) and (*d*) are of flowers pulsed with disodium fluorescein, sectioned to 0.5 mm-thick and viewed under UV illumination. The scale bars represent 100 μm in (*a*) and 500 μm in (*b*), (*c*) and (*d*). ep = epidermis, ft = floral tube, p = pedicel, sc = separation cavity, v = vascular tissues.