Flora 233 (2017) 99–108

Contents lists available at ScienceDirect

Flora

journal homepage: www.elsevier.com/locate/flora

Hydathode pit development in the alpine plant *Saxifraga cochlearis*

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ARTICLE INFO

Article history:
Received 28 August 2016
Received in revised form 12 May 2017
Accepted 29 May 2017
Edited by Alessio Papini
Available online 1 June 2017

Keywords:
Hydathode
Leaf
Pit
Pore
Lobe
Epithem

ABSTRACT

The genus *Saxifraga* contain many species that form a calcified crust on the leaf surface, originating from pore-containing pits that form part of the leaf hydathode structure. The detailed morphology and development of the hydathodes are not well understood for this genus. We present a study of the fine structure and developmental stages of hydathode pit formation along the leaf margin of the alpine plant *Saxifraga cochlearis* and cryo-fracture to reveal the internal hydathode structure. Raman- and stereo-microscopy have been used to deduce the composition and distribution of the crust. We find the pits occur as a developmental series along the leaf where conserved and oriented divisions within leaf lobes appear to give rise to the early pit. Both pit formation and lobe maturation are linked. As the pits deepen, hydathode pores differentiate to thick-walled, cone shaped structures and, together with the ovoid epithem tissue extrude liquid resulting in deposits of calcite that fill the pits and spill on to the leaf margin. The epithem does not possess the typical organisation or cell morphologies that have been reported for hydathodes from other plants, lacking lobed cells and having an indistinctive sheath-like cell layer.

1. Introduction

The hydathode, sometimes referred to as a "gland", permits the expulsion of potentially large amounts of fluids to the leaf surface. This process, termed guttation, has been studied for over 300 years. Proposed functions of hydathode-mediated guttation include regulation of internal water pressure, maintaining mineral ion homeostasis, offloading of harmful substances and protection against herbivory and pathogens (Singh, 2014; Nazrul Islam and Kawasaki, 2015). In plants such as many *Saxifraga* species, the guttation gives rise to encrusted leaves. This genus contains in the order of 400 species, its taxonomy has been constantly expanded and refined, with morphological resources available for many species (Gornall, 1987). It is a holoarctic genus occupying habitats ranging from the arctic tundra to shady rock faces on mountains in southern Europe. A collection of European Saxifraga species, set up as resource for study over 50 years ago, is held at the Cambridge University Botanic Garden (Webb, 1964).

Saxifraga cochlearis is a perennial silver saxifrage in the section Ligulatae that was first documented in 1832 by Reichenbach. It has a narrow distribution in the wild, growing only in two locations; the Tende mountains and a small area on the Portofino Peninsula in Italy (McGregor, 2008). *S. cochlearis* grows in shady rocks generally on limestone forming a cushion of encrusted glaucous rosettes all branching from a central root system. It has oblong leaves that widen with greater distance from the petiole, closing to a blunt tip (Webb and Gornall, 1989). Flowering occurs from late May and, compared to its Ligulatae relatives, it is one of the last to flower. Ultrastructural studies that make use of modern microscopy-based techniques have not been applied to this species and even with other saxifrages such analyses have focused upon their reproductive structures (Ladinig and Wagner, 2009; Konarska, 2014; Gornall et al., 2015). We are specifically interested in the development of the prominent hydathodes arranged in a line along the entire leaf periphery, resulting in large "spots" of easily visible white crust. Their basic anatomy and physiology were the subject of several studies between 75 and 150 years ago (Waldner, 1877; Engler, 1919; Kurt, 1930). Many Saxifrage hydathodes are loosely termed "chalk glands" due to the apparent deposition of a white crust around the pores and extending across some of the leaf surface and margin. The crust of *Saxifraga* species is predominantly made up from calcium carbonate (Unger, 1861) and, across the genus, exhibits variation in crystallite grain morphology and the presence of other constituents such as magnesium carbonate and trace metals (Kurt, 1930). Plants such as select Saxifrages make use of a passive hydathode system that links surface pores with the vascular network (Ivanoff, 1963). It is known from *Caltha palustris* anatomical observations that the pores appear to start out as guard

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http://dx.doi.org/10.1016/j.flora.2017.05.018

mother cells, form a stomatal-type pore and then the guard cells somehow become specialised, forming a rigid and wide-open space to allow the passing of the guttation fluid (Stevens, 1956). Limited data exist on the events leading to specialisation of epidermal regions that contain the future pores. An early observation, from *Saxifraga cuneifolia*, found that the pore (guard) mother cell forms in the centre of, and surrounded by, a population of much smaller epidermal cells (Waldner, 1877). The mature pore allows the free passage of the guttation fluid from the small intercellular spaces of the epithem tissue below (Haberlandt, 1914). The epithem is a specialised parenchymous organ with cells that are morphologically distinct from the surrounding leaf tissue. The epithem is discernible after the guard cells of the future water pore have formed at the epidermis (Mortlock, 1951). Studies from various plants find the epithem to develop as a mass of non-uniform, commonly lobed cells and surrounded by a distinctive sheath cell layer (Lersten and Curtis, 1991 Galatis, 1988; Chen and Chen, 2005; Chen and Chen, 2007).

The aims of this study are to establish a detailed account of the morphology, organisation and development of the hydathode from an encrusted *Saxifraga* species and to compare with observations from other non-related plants. Due to its amenability to cryofracture, coupled with hydathode distribution as an entire developmental series along the leaf margin, we have focused upon *S. cochlearis*.

2. Material and methods

2.1. *Propagation and growth of* S. cochlearis

Plant samples were grown at the Cambridge University Botanic Gardens. They are from plants originally collected in the wild and grown in a well-drained gritty peat-free compost, supplemented with a slow release fertilizer, in terracotta pots that were plunged in sand beds to maintain correct moisture levels. For propagation, single rosette cuttings were taken generally after flowering and grown on in a free draining loam and peat-free growing medium. To protect from excessive rain, plants were grown under an open sided polyurethane canopy and watered by hand using collected rain water. All data are from one accession of *S. cochlearis*.

2.2. *Cryo-scanning electron micoscopy (cryoSEM)*

The principle of cryoSEM is described and reviewed by Sargent (1986). Between 3 and 5 leaves, taken within the top half of the *S. cochlearis* rosette, were careful removed with forceps and placed side-by-side flat on a brass stub, stuck down with a cryo glue preparation consisting of a 3:1 mixure of Tissue-Tec (Scigen Scientific, USA)) and Aquadag colloidal graphite (Agar Scientific, Stansted, UK)) and then plunge frozen in liquid nitrogen with vacuum applied. For sample preparation for cryofracture, up to 3 leaves at a time were placed vertically in recessed stubs held by the cryo glue preparation. Frozen samples were then transferred under vacuum to the prep chamber of a PT3010T cryo-apparatus (Quorum Technologies, Lewes, UK) and maintained at −145C. For cryoSEM without fracture, surface ice was removed using a sublimation protocol consisting of −90C for 3 min. Where there was excessive surface ice samples were discarded and cryo-preparation repeated. For cryo fracture (i.e. non-surface imaging), no sublimation was required and instead a level semi-rotary cryo knife was used to randomly fracture the vertically oriented leaves. Fractures were repeated for sets of leaves until fractures that included the hydathode region were obtained. All samples were sputter coated with platinum until a measured thickness of 5 nm was recorded. Samples were then transferred and maintained cold, under vacuum into the chamber of a Zeiss EVO HD15 SEM fitted with a cryo-stage. Images were taken on the SEM using a gun voltage of 6 kV, I probe size of 460 pA, a SE detector and a working distance of between 5 and 6 mm.

2.3. *Raman microscopy*

Collection of Raman point spectra and spectral mapping, from leaves collected from the top-half of the rosette, were carried out on a Renishaw InVia Raman microscope fitted with a Leica 20× objective and a 532 nm laser with power level set to 50%. The spectral collection window was centred around 750 cm^{-1} and acquired using a 1 s exposure, 3× accumulation. Images were plotted as signal intensity at 1086 cm^{-1} to visualise calcite deposits and a 1086:1156 cm-1 ratio map for calcite thickness.

2.4. *Stereomicroscopy*

Stereomicroscope images were taken with an Olympus SZ61 fitted with an LED ring light and a Nikon D5200 SLR camera. For mapping the calcite reflection, images were converted to greyscale using ImageJ (Schneider et al., 2012) and then shown as the "Fire" lookup table where white pixels are shown as red/orange.

3. Results

3.1. *The hydathode pit viewed by cryoSEM*

Leaves from *S. cochlearis* were processed for cryoSEM. Although the leaves are commonly encrusted, some areas appeared to be cleared of the crust after cryo-processing allowing observations of the epidermis. The upper surface of the distal portion of the leaf, that is the region furthest away from the petiole, possesses deep pits near the margin (Fig. 1A, arrow). These pits are commonly encrusted, sometimes heavily (Fig. 1B). A cleared pit, only lightly encrusted in places, is shown in Fig. 1C where the leaf epidermal cells are continuous with the steep sides and a pore-type structure is seen in a flat area at the bottom. Despite sublimation, the pore exit contains a block of ice, whereas no ice is observed in any other part of the pit, demonstrating that a relatively large body of water was in the pore at the time of freezing. These properties identify the pore has a hydathode exit point undergoing guttation.

3.2. *Pit development*

When looking progressively from the petiole to the leaf tip, the pits appear to occur as a developmental series, becoming larger and deeper (Fig. 2A, the petiole is at the top of the image). We can use these series in order to understand how pits and pores form in this species. SEM images taken close to the petiole show two stomatal-like pores surrounded by small cells, occurring close to a lobular margin-tooth composed of 6–8 cells (Fig. 1A arrowheads, Fig. 2B and C). Further away and later in development, a distinct flattened recess has formed and one set of guard cells appear to be part of the mature pore (Fig. 2D). The remaining stomatal-like structure is not visible, coincident with encrustation (Fig. 2E) that becomes heavier as pits deepen (Fig. 2F).

For understanding cellular organisation prior to pit formation, we looked at the petiole of a young leaf (Fig. 3A). The future pit region consists of smaller cells relative to the rest of the leaf. An exception is a large oval-shaped cell (Fig. 3B, arrow) that sits within this pit region. A cell more distal to the leaf margin becomes enlarged (circled red) and, together with its partner, have assumed locations consistent with the future hydathode pores. A neighbouring pair of cells exhibit retarded growth (circled green) or undergo