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Leaf margin organisation and the existence of vaterite-producing hydathodes in the alpine plant *Saxifraga scardica*

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ABSTRACT

Some sections of species within the genus *Saxifraga* deposit a chalky crust on the surface of their leaves, originating from the guttation medium produced by the sunken hydathodes found generally at or near the leaf margin. The organisation of the hydathode tissues, that of the rest of the leaf and the physiology of the crust is poorly understood. We have used cryo-SEM and cryo-fracture to study leaf tissue organisation and structure in *Saxifraga scardica* and compared it to the imaging data with the previously characterised *Saxifraga cochlearis*. We find *S. scardica* contains a transparent and tapered leaf margin containing thick walled cylindrical cells that may serve to deflect light to the adjacent palisade mesophyll tissue. Raman microscopy reveals the *S. scardica* leaf crust contains the rare and metastable calcium carbonate polymorph vaterite whereas the crust from *S. cochlearis* contains only calcite. Vaterite-crust is also observed on the leaves of some species within the section *Porphyrion* but is not found on members of the section *Ligulatae*. The implications of these findings are discussed.

1. Introduction

The genus *Saxifraga* comprises approximately 400 species, arranged within sections and subsections based on molecular and morphological characteristics (Gornall, 1987a). Those species comprising encrusted leaves as a result of guttation from hydathode structures termed “chalk glands” are found in sections *Ligulatae, Xanthizoon* and *Porphyrion* (for detailed discussion and molecular taxonomic analysis of the *Saxifraga* see Conti et al., 1999 and Tkach et al., 2015). The leaves of these and other sections are commonly small and fleshy. Information on *Saxifraga* leaf anatomy is often patchy. Detailed drawings of arctic *Saxifraga* have given an insight in to epidermal and, to some extent, mesophyll organisation (Galløe, 2010). *Saxifraga* leaves appear to have dorsiventral mesophyll organisation and a thick leaf cuticle as demonstrated for *Saxifraga hirculus* and *Saxifraga paniculata* (Codignola et al., 1990; Pyankov et al., 1999). Alpine *Saxifraga* species generally have a defined visible boundary between palisade and spongy mesophyll whereas for arctic species the distinction is less clear (Galløe, 2010). For alpine desert plants, the dorsiventral organisation of mesophyll is speculated to aid photosynthesis at high irradiation but at a cost to decreased drought tolerance (Pyankov et al., 1999). It is known that hydathodes consist of thin walled epithem tissue and, where they are found to be sunken within the leaf, extrude guttation medium to fill the pit and adjacent leaf margin area as observed for *Saxifraga cochlearis* and *Saxifraga crustata* (Gardiner, 1881; Wightman et al., 2017). In *S. paniculata* (synonym *S. aizoon*), the plastids of epithem cells have been shown to contain electron dense structures of phytoferritin and may be the principal site of synthesis or storage within the plant (Perrin, 1970). Encrusted species, as a rule, tend to possess these pits, often termed “cavities” whereas non-encrusted *Saxifraga* species have hydathodes positioned close to the leaf surface (Galløe, 2010). Some non-encrusted species instead contain internal calcium oxalate structures termed foliar crystals that may have an analogous role to the crusted hydathodes (Gornall, 1987b). For *S. cochlearis* the crust consists of the calcium carbonate polymorph calcite (Wightman et al., 2017). Leaf trichomes, where observed, tend to possess multiseriate morphology for the encrusted species (Gornall, 1986). A thorough comparative analysis of leaf venation patterns show camptodromous veins within the encrusted species where the open vein system has been proposed to be well adapted to extruding the calcium salts (Zhang et al., 2015).

Saxifraga scardica is a member of the section *Porphyrion*. Its name originates from where it was discovered, in 1839, by Grisebach on the two main peaks of the Sar Planina in the Scardus mountain range of the Balkans. Its distribution sees it extend though northern Montenegro, Kosovo, Albania and in to Greece. It commonly grows at altitudes between 1800 and 2500 m on limestone rocks in both alpine and subalpine zones. It reaches altitudes as high as 2900 m on sunny positions of Mount Olympus to as low as 400 m in nearby ravines and the Treska

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gorge (Strid and Tan, 1986; Webb and Gornall, 1989). *Saxifraga scardica* forms a compact and dense cushion that over time can form hummocks or low mats. The stiff and spiny rosettes are 10–15 mm in diameter and leaves are 5–15 mm x 2–4 mm lanceolate to spathulate with a tip described as acute/mucronate. The leaves are fleshy and vary in colour from dark to light green and the surface of the leaf is convex with the tip being distinctly keeled inwards. It has been documented that the *S. scardica* leaves have "translucent margins" (Webb and Gornall, 1989) and further described as having "cartilaginous edges" (Irving and Malby, 1914; Smith and Fitch, 1909). Hydathodes occur at the edge of the leaf and number between 9 and 15. The inflorescence consists of cup shaped flowers which flatten as they open. The petals are commonly conspicuous 7–12 mm obovate, overlapping to slightly separate and are white sometimes fading to pink, however, the shape and colour of these petals as well as other attributes can vary (Horný et al., 1986).

Given that *S. scardica* is found in a distinct section compared to the recently characterised *S. cochlearis* and appears to have key differences in leaf morphology, we examined the ultrastructure, composition and physiology of *S. scardica* leaves with a focus on the hydathodes and transparent margin. We report notable differences between the two species that include the crystal structure of the crust and the precise arrangement of the mesophyll. We extend the molecular analysis of the crust to other *Saxifraga* species. We demonstrate the *S. scardica* margin is a unique tissue with light deflection properties that may serve to aid light capture by the leaves.

2. Materials and methods

2.1. Propagation and growth of studied Saxifraga species

The *Saxifraga* used in this study were (i) section *Porphyrion* species *S. scardica* Griseb., *S. diapensioides* Bellardi, *S. sempervivum* K. Koch, *S. ferdinandi-coburgi* Kellerer & Sünd. and (ii) section *Ligulatae* species *S. cochlearis* Rchb., *S. longifolia* Lapeyr., *S. valdensis* DC., *S. paniculata* Mill., *S. callosa* Sm. var. *australis* (Moric.) D.A. Webb and *S. crustata* Vest. All plant samples were grown at the Cambridge University Botanic Garden (52.1935°N, 0.1258°E), in terracotta pots containing a loam based peat free John Innes compost with extra grit. The pots have been plunged in sand beds to maintain correct moisture levels. For propagation, single rosette cuttings from post-flowering plants were grown in a mix of equal parts sharp sand, grit and perlite. Plants were grown under an open sided polytunnel and watered by hand using collected rain water. Care was taken to avoid excessive exposure to the sun as recommended by Harding (Harding, 1981). Leaves were harvested and observed in June for cryoSEM and light microscopy. For Raman analysis of the crust, leaves were harvested according to the schedule in the legend of Table 1. Leaves for all analyses were taken from non-flowering stems at a developed stage intermediate in size between the youngest leaves visible at the apex and the first fully grown leaves in the upper half of the rosette.

Table 1
Survey of crust polymorph composition in *Saxifraga* species revealed by Raman microscopy. Samples were taken and assayed at three time points: April/May 2016 (Assay 1), March 2017 (Assay 2) and May 2017 (Assay 3, for confirming vaterite-containing leaves).

Saxifraga species	Assay 1	Assay 2	Assay 3
S. cochlearis	C	C	C
S. scardica	VC	VC	VC
S. longifolia	C	C	nd
S. valdensis	C	C	nd
S. paniculata	C	C	nd
S. diapensioides	V	V	VC
S. sempervivum	V	V	V
S. ferdinandi-coburgi	V	VC	VC
S. callosa	C	C	nd
S. crustata	C	C	nd

C = Calcite, V = Vaterite, VC = Vaterite/Calcite mixture, nd = assay not done.

2.2. Stereo, light and confocal microscopy

Stereomicroscopy (Fig. 1A, B) was carried out using a Zeiss V20 stereomicroscope fitted with a 1x PlanApo S objective and a Zeiss AxioCam HRc camera. Uniform illumination of samples was achieved using a Schott ring light from above and a gooseneck light pair from the sides. Axiovision software (Zeiss) was used for acquisitions that comprised a Z-stack that encompassed the entire focal depth of the sample followed by the Extended Depth of Field plugin for producing a single in-focus image from the z-stack. Transmitted light microscopy of leaf margins (Fig. 3A, B) used a Leica DM1000 microscope, 20x objective and a Leica ICC50 W camera. Confocal microscopy (Fig. S4) for imaging ultra-violet (UV) autofluorescence was carried out on hand-cut transverse sections of leaves mounted in water with a coverslip and viewed using an upright Leica SP8 confocal microscope fitted with a 20x dry objective lens. The 405 nm laser was set to 28% and the detection range was set to 420–551 nm (blue/green) and 646–697 nm (red). The transmitted light channel used the 561 nm laser set to 9%. Two leaves were sectioned and imaged, one is shown in Fig. S4 and the other gave the same result.

2.3. Cryo-scanning electron micoscopy (cryoSEM)

Leaves (3x *S. cochlearis* and 3x *S. scardia*) were arranged and observed in paired sets, one from each species, on cryo-stubs ready for cryoSEM preparation. CryoSEM was carried out according to the protocol described in Wightman et al. (Wightman et al., 2017) with the following modifications: (i) The time between harvesting the *S. scardica* leaf and plunge freezing in the liquid nitrogen was less than 5 min to avoid the undesirable drying of leaf internal tissues. (ii) The Zeiss EVO HD15 SEM used either the secondary electron (SE) detector, with 4–5 nm of platinum coating to enhance contrast, or the backscattered electron detector (BSD, with 1.5–3 nm coating to eliminate sample charging. (iii) The accelerating voltage was set to 8 kV (SE imaging) or 25 kV (BSD imaging). The I probe was set to 2.4 nA for both forms of imaging.

2.4. Raman microscopy

Raman point spectra and map data were produced from encrusted leaves found close to the vegetative stem apex, using a Renishaw InVia Raman microscope fitted with a 50 x dry objective and a 532 nm laser. Leaves were placed on a stainless steel slide with no coverslip. Point spectra (Fig. 2A and Table 1) were generated after 1 s exposure time, 3x accumulation, laser power of 50% and a spectral centre set to 750 cm^{-1} and pinhole engaged. Raman mapping used the StreamlineHR function of the InVia with the 532 nm laser set to 5%, 0.8 s exposure, no accumulation, pinhole disengaged and a spectral centre set to 750 cm^{-1}. Raman analysis of the transparent leaf margin had the 532 nm laser set to 50% power, 4 s exposure, 3x accumulation and spectral centre set to 1250 cm^{-1}. Raman peaks were assigned by comparing to the reference assignments reported by Schulz and Baranska (2007).

2.5. Comparisons of cryoSEM and Raman data with Arabidopsis interfascicular fibres

The same cryoSEM and Raman apparatus used for *Saxifraga* research is used extensively for analysis of cell wall ultrastructure and chemistry in the secondary wall forming tissues of the xylem and fibres in the model plant *Arabidopsis thaliana* Heynh. (R. Wightman, unpublished data) and this work provided the link in terms of the striking morphological similarities between *S. scardica* margin cells and *Arabidopsis* fibres. Stems from *Arabidopsis* ecotype Landsberg *erecta*