RESEARCH ARTICLE

An Enzymatic method to Isolate Salt Gland from the leaves of *Acanthus ilicifolius* L. for Structural Characterization with Auto-fluorescence Imaging and Inorganic Ion Concentration Studies

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Abstract

Excess salt regulation, an intricate mechanism in halophytic plants, has long fascinated researchers. In this paper, we focused on a typical mangrove-associated plant *Acanthus ilicifolius* L. (family-Acanthaceae), to investigate the structural organization of its salt glands. The investigation encompassed a multi-faceted approach involving enzymatic isolation, ultrastructure studies under electron microscopy (SEM) and light microscopy, autofluorescence activity assessment, and analysis of inorganic ion concentrations. We applied an enzymatic method to isolate individual glands from embedded epidermal salt glands. Isolated glands and epidermal peels were stained with safranin and toluidine blue and ultrastructural detailing was done using light microscopy and scanning electron microscopy. Analysis of the leaf ion concentration was done by flame photometry and cellular inorganic ion content by X-ray microanalysis (Edax). The multicellular asterid-type gland consists of eight secretory cells, three stalk cells, and basal cells. The glands are located on both of the leaf epidermis and emit yellowish-green fluorescence light from secretory cells. The cells showed an accumulation of high concentrations of inorganic ions such as sodium, chloride, calcium, and potassium under high salinity (17.5–19.5 ppt) stress conditions. Therefore, this study concentrated on the structural composition, ultrastructure, autofluorescence activity, and ion concentration of the salt glands (SGs) in the transverse section. In this study, the first on *A. ilicifolius* salt gland, a holistic understanding of the glandular architecture, and its complicated cellular structure have been examined more comprehensively.

Highlights

- Salt glands (SG) secrete the excess salt from salt-stress *Acanthus ilicifolius* L. leaves, which maintain the cellular turgor pressure and ion homeostasis.
- The multicellular asterid-type salt gland consists of secretory cells, stalk cells, and basal cells. The secretory cell wall is composed of lignins and cutin that make autofluorescence light (yellowish-green).
- Salt glands (SGs) are composed of inorganic ions such as calcium, magnesium, sodium, chloride, potassium, and phosphorus, which facilitate osmotic and ionic regulation.
- Successfully isolated the salt glands (SGs) by advanced enzymatic methods for clear visualization of the glandular architectures.

Keywords: Autofluorescence, Edax, Gland isolation, Secretory cell, Ultrastructure.

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Introduction

Coastal regions are renowned for their diverse and unique ecosystems, where plants must overcome a myriad of challenges, including high salinity levels. Halophytic plants mainly grow in high salinity or black water and adapt to this harsh environment. They have many adaptive features, such as specialized salt glands located on the epidermis of the stem and leaves (Arisz *et al.,* 1955; Fahn, 2000; Thomson *et al*., 1988). Some of the halophytic plants secrete salt through their leaf epidermal glands (Thomson & Liu, 1967). There has been research demonstrating that plants excrete salt in saline solutions under field and controlled conditions (Zauhaier *et al*., 2015). Leaf surfaces excrete the salt that crystallized after transpiration and became visible even to the naked eye (Gulzar *et al*., 2003; Barhoumi *et al*., 2007b). Various studies have demonstrated that excretion is mainly composed of inorganic ions such as sodium, calcium, carbonate, chloride, magnesium, nitrate, phosphate, potassium, and sulfate (Thomson, 1975).

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Moreover, lanthanum, rubidium, and silicon may also be involved (Sakai, 1974; Thomson *et al.,* 1969; Thomson, 1975). Usually, this gland-excreted salt composition mostly depends upon the root environment and surrounding humidity, temperature, and light intensity (Ding *et al.,* 2010; Drennan & Pammeter, 1982; Gorham, 1987; Imada *et al.,* 2012; Liphschitz & Waisel, 1974; Marcum & Murdoch, 1992; Pollak & Waisel, 1970; Pollak & Waisel, 1979; Scholander *et al.,* 1962). The excretion of salt, which is a natural adaptation to high salinity, may be an excellent approach for exploring the mechanisms behind this adaptation. To date, however, only a few studies have been done and the excretion mechanism in plants but it is still not clear, and only a few hypotheses have been suggested. Earlier studies on ultrastructural studies of glands and some studies on leaf histology and anatomy (Balsamo & Thomson, 1993; Barhoumi *et al*., 2008; Drennan *et al.,* 1987; Faraday & Thomson, 1986a; Faraday & Thomson, 1986b; Fineran., 1985; Shimony *et al.,* 1973; Thomsom *et al.,* 1988) have been conducted. *Acanthus ilicifolius* L., a notable mangrove-associated plant species, thrives in such adverse environments and exhibits fascinating adaptations to cope with salt stress. Salt glands, also known as secretory structures, serve as vital organs that facilitate the excretion of excess salts, allowing *A. ilicifolius* L. to maintain a delicate ion balance essential for its survival in saline-rich habitats. However, there is currently very few researches available and ultrastructural studies with their patterns and intensity of autofluorescence in plants remain unclear, with just a few hypotheses having been proposed (Deng *et al*., 2015; Drennan & Pammenter, 1982). Further research is needed on salt excretion, especially on the structure, pattern and inorganic ion accumulation in plant salt glands. Therefore, we are investigating the ultrastructure of *A. ilicifolius* salt glands, and their functioning mechanisms under natural habitat, to improve understanding of salt excretion. In addition, we examine the intricate ultrastructural details of these salt glands using advanced microscopy and imaging tools.

Materials and Methods

A. ilicifolius leaves were collected from the Digha Mohona seashore line toward the Champa River adjoining the Bay of Bengal, 21°38.426′ N & 87°33.557′ E on 08/04/2022. The plant was preserved as a Herbarium (VU/Arpita/0057/09) [Vidyasagar University Herbarium] for proper identification. Collected plant leaves were washed with dH_2O and then fixed in FAA solution for further examination. Three strategies were followed for the observation of salt gland micromorphology and anatomy.

Light microscopy

Fresh leaf samples were cut into pieces (2cm×2cm) and fixed with 3% sodium hydroxide or potassium hydroxide pellet in distilled water by keeping in a water bath (50°C) for 5 minutes. Then peel off the upper and lower epidermis and examine under a light microscope (Leica D3000 & Zeiss primo star) after washing and staining with safranin and toluidine blue.

Fresh leaf Transverse section (TS) was cut with a cryo-microtome and the section was bleached with 0.5% sodium hypochlorite to clear other cellular debris. Then a clear structural vision of salt glands and better intensity of autofluorescence of SGs under a light microscope (Leica D3000 & Zeiss primo star).

SEM and Energy Dispersive Analysis of X-rays (EDAX)

The leaf epidermal segment (5 mm) was dehydrated with different series of alcohol and air dried for 12 hours. The samples were mounted on metal stubs using sticky black tape, which increased conductivity. Gold-containing glue was coated for even more conductivity and an accelerating voltage of 10 to 20 kV. Ultrastructural studies of the salt-secreting gland were conducted with an energy-dispersive analysis X-ray in SEM (Zeiss supra-40). Both leaf epidermal surface and were scanned gland three-dimensional image and its surface area were observed.

Salt gland isolation and purification

We applied a modified enzymatic method of gland isolation from the leaf epidermis (Tan *et al*., 2010) and studied the inorganic ions contained in the glands. Fast of all collected leaves were treated with 3% potassium hydroxide pellet (KOH) and heated at 70°C for 5 minutes. Then, the epidermal layer was removed and treated with 20 mM sodium hypochlorite at min for chlorophyll removal. These epidermal layers were cut into pieces (2 × 2 cm) and fixed with an enzyme mixture (pH 5.7) consisting 0.2% (W/V) pectinase (Sigma) and 1% cellulase (Sigma). Collected fresh leaves were cut into pieces (2 × 2 cm) and fixed with an enzyme mixture (pH 5.7) 0.3% (W/V) pectinase (Sigma) and 2% cellulase (Sigma). This mixture was vacuum infiltrated for 10 min and then, incubated in the dark at 30°C, 30 rpm for 1-hour in a BOD incubator with a shaker. To differentiate upper epidermis from lower epidermal peels and the mesophyll-palisade layers were gently scraped off using a scalpel before the peels did not have chlorophyll-containing cells. These peels were ground in a mortar to release the separated gland from the epidermal cell. For more salt glands to be released the grinding process was repeated using fresh 1X PBS buffer (0.1 mM) (pH 7.4). The suspensions were collected in 1.5 mL falcon tubes and centrifuged (7000 rpm) at 4°C for 5 minutes. The collected glands were rinsed with 2 mL of 1X PBS buffer (pH 7.4) and kept on ice for subsequent observations. The larger cell clusters and peel the debris were removed. The glands were observed under a phase contrast compound microscope (Zeiss Primo star). We transferred the mixture to a mortar containing 3 to 5 mL of 1% PBS buffer (pH 7.4).

Measurement of ion concentration by flame photometry

Fresh plant leaves are collected from natural habitats, washed with distilled water and dried. Over 1 g of powder sample was placed in a digestion tube with 15 mL of nitric acid and kept for 12 to 24 hours at room temperature. After mixing, 2 mL of Perchloric acid and 4 mL of Sulphuric acid were added to the sample and kept in the muffle furnace for digestion. Gradually increase the temperature from 50°C to 250 to 300°C. Approximately 70 to 85 minutes after digestion, the solution was cooled. The mixture was transferred to 100 mL volumetric flasks and diluted with dH2O. Then extracts were analyzed for ion content (Na+, Cl- and K+) by flame photometry.

Results and Discussion

Light Microscopy and Autofluorescence Studies

Multicellular asterid-type SG is found in the family Acanthaceae (Santose *et al*., 2016). Multicellular asterid-type glandular trichomes are present on both surfaces of *A. ilicifolius* leaf blades (Figs 2C-F). The structure of the SG is composed of three types of cells viz. Secretory cells, stalk cells and basal cells (Figs 2D-F). The mature glandular head (secretory head) consists of an 8-radially arranged secretory cavity (Figs 1E-O) with a single epidermal layer (Figs 2A-F). The three stalks are short, being composed of a single rectangular cell with hyaline cytoplasm. The basal cell is thin-walled, isodiametric, and vacuolated (Figs 2A, B). The gland is present on both epidermal surfaces (Fig 1F-L & 2A, B). Gland frequency on both surfaces is in the range of 9–12 glands cm^2. We are examining more glands are present on the upper epidermis than the lower epidermis and more active than in the lower leaf surface (Fig 1F, L). The glands comprise eight (8) secretory cells (Sec) (length 20.37 µm), three stalk cells, and one large basal cell (collecting cell). Basal cells are separated from leaf palisade cells by small sub-basal cells or cuticular cells. The gland top and sides clearly view comprise a thick cuticle layer continuous with the whole leaf surface. This cell is located in a small depression on the leaf surface, slightly below the level of the other epidermal cells (46.875 × 18.75 µm in size). Short-stalked glands are ubiquitous throughout the Acanthaceae family (Bhogaonkar & Lande, 2015; Immelman, 1990), and *A. ilicifolius* L. has a short three-stalk cell, (Fig. 2E-J, L). The SG (glandular hair) of Acanthaceae is also very much similar to the trichomes of Lamiaceae, which secrete essential oils (Shimony *et al.,* 1973). *A. ilicifolius* leave salt gland size (49.13 µm) is larger than that of *Avicennia* sp., *Tamarix* sp., *Limonium* sp., or *Aegialitis* sp. and smaller than that of *A. corniculatum* (Atkinson *et al.,* 1967; Campbell & Strong, 1964; Cardale & Field, 1971; Drennan, 1987; Tan *et al.,* 2010; Thomson & Liu, 1967). These glandular trichomes have glandular-headed secretory cells along with a subcuticular space where oils containing volatile secondary metabolites are accumulated. They have a basal cell embedded in the epidermis, a two-celled stalk, and one basal cell beneath the epidermis (Fig 2C-F) (Ascensao *et al.,* 1995; Giulini and Bini, 2008; Serrato Valenti *et al.,* 1997; Werker *et al.,* 1993). A secretory SG is typically located on the leaf surface, usually in a pit or depression. Salt can probably be secreted efficiently into these depressions because dew accumulates in them. The density of SG varies from species to species. Salt gland diversity depends on the species and leaf age. *Limonium* sp. and *Zoysia* sp. have gland densities of 20 to 50 mm^2 (Ding *et al.,* 2010a; Yamamoto *et al*., 2016). It is also possible that soil salinity and leaf age affect the structural integrity of salt glands. For example, *Porteresia coarctata* salt hairs tend to burst when soil salinity rises, whereas their more elongated abaxial counterparts increase (Sengupta & Majumder, 2009). In the transverse section, leaves are isolateral, and mesophyll tissue is compactly arranged and has a secretory cavity line with the epidermal layer. The glands are present on the leaf adaxial (upper) and abaxial (lower) epidermis, but stomata (diacytic) are present only on the lower surface (Fig- 1F-P). A thick cuticular layer overlies the normal cell wall (Franke, 1967). Microtome sections of paraffin-embedded leaf specimens reveal collecting cells beneath the stalk cells of each salt gland (Fig 2C-F). Because whole-mount specimens have limited light penetration of intact leaves, leaf surface clearing treatments are required for direct observation of salt glands. In addition, the glands are intrinsically fluorescent, so they can be optically sectioned without fluorescent dyes and constructed into two-dimensional images. In addition, the investigation extends to the autofluorescence activity within *A. ilicifolius* leaves. Autofluorescence is a natural phenomenon in which certain cellular components emit fluorescent light when exposed to specific wavelengths. Blue light excited the leaf's upper epidermal surface and then emitted green autofluorescence (500 nm) light from the epidermal wall (Lichtenthaler & Schweiger, 1998), gland membrane and secretory cavity. Cuticular envelopes, secretory cells, and stalk cells were most commonly autofluorescing. The outer periphery of nearly all secretory cells was shown to have a greater intensity of green autofluorescence by optical sections. It was observed that secretory cells, but not stalk cells, appeared to fluoresce when spherical or irregularly shaped objects were present. According to the three-dimensional view of the SG taken from a top view, which consists of 8 secretory cells (Sec) and 2 stalk cells (Fig 1P-U). Lignin, which contains fluorescein, makes up the majority of the cell wall.

Gland Isolation

This method proved to be a less invasive imaging method which helped in preserving cellular and tissue health. This study involved enzymatically isolating the glands from leaf epidermal peels and examining them using microscopic imaging (Fig 2G-L). This isolation method minimizes interfering epidermal and mesophyll-palisade cell layers and a large number of salt glands could be isolated easily. After 1-hour of enzyme digestion, mesophyll palisade tissues were easily distinct from the adaxial (upper) and abaxial (lower) epidermal surfaces of leaves (Fig 1C) and incubated in an enzymatic mixture with 1X PBS buffer (Fig 1D). To reduce the contamination with other cell types before salt gland isolation, mesophyll-palisade cells, which exhibit a green color due to chloroplasts, can be easily removed beneath the upper epidermal peels. The isolated glands (54.43 × 63.36 µm in size) were visualized under a phase contrast microscope. Large secretory cells (33.16 × 13.11 µm), basal cells (10.5 µm in size), and 3 stalk cells (9.99 µm in size) were prominently present. The cuticular cavity of the individual isolated gland (Figs 2G-L) was also visualized.

Electron Microscopic Studies

Scanning electron micrographs as presented in Figs 2M and R showed evident morphological differences between both leaf surfaces. Salt excretion was observed in natural habitats with a salinity of around 18 to 20 ppt and pH of 5.8 to 7. The leaf's upper surface is rough and corrugated and has numerous glandular trichomes (salt gland) (Fig. 2 N), while the surface is smooth on the lower side and contains stomata and glandular hair (Figs 2P-T). Salt glands were embedded within the leaf epidermal cells. The upper surface glandular trichome frequency is more active than the surface of the abaxial part (Figs 2M, N). Glands are encrypted within the epidermal layer, and large secretory cavities encircled by a single layer of barrel-shaped, thin-walled epithelial cells occur in the upper mesophyll zone in the leaves

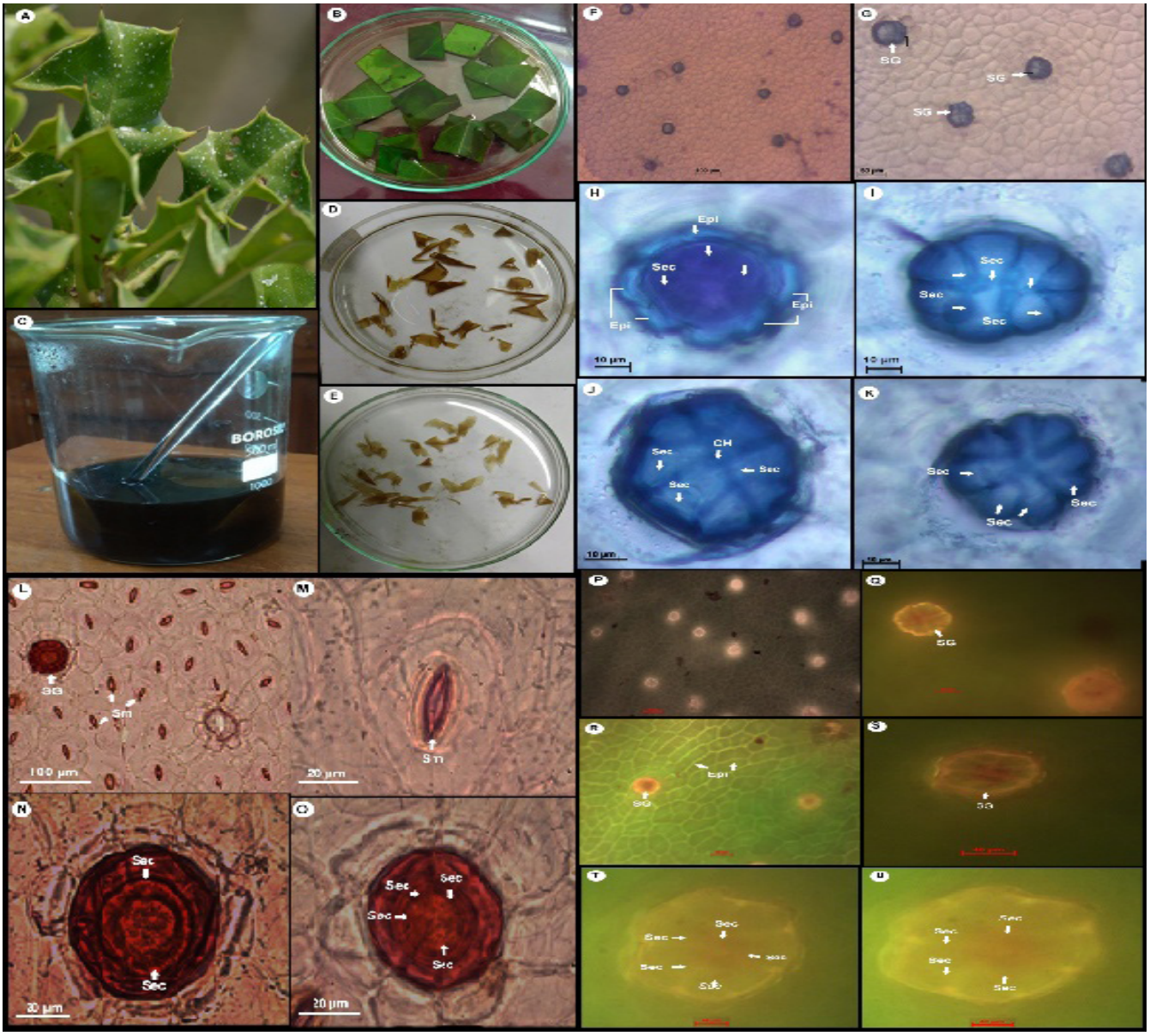

Fig. 1: A- secreted salt crystal on leaf surfaces; B- enzymatic treatment of fresh leaf pieces; C- leaves treated with 3% of KOH pellet. D- Epidermal peel treated with 20mM sodium hypochlorite; E- Epidermal peel fixed with enzyme mixture. Light microscopy images and stain with Toluidine blue. (F-K) Salt gland with 8- secretory cells (Sec- Secretory Cell, SG- Salt Gland, and CH – Central Hole & Epi – Epidermal cell). Light microscopy images and stain with safranin (L-O). L- Leaf Abaxial surface; M- stomata; N- Secretory cavity (adaxial); O- salt gland (abaxial). (Sm- stomata, SG- salt gland, Sec- secretory cavity). Light microscopy images (Auto-fluorescence activity) (P-U) - salt gland (fluorescence light emission). (Sm- stomata, SG- salt gland, Sec- secretory cavity).

(Figs 2P, S). The accumulation of salt on the leaf surfaces was observed, which are cube and sphere-shaped crystals at the former (Figs 2M-O). This gland has a very compact and complex structure compared with other species. Various shapes of salt crystals were observed on the leaf epidermis (Fig. 2O) that gathered around the gland surface, as observed under high magnification by SEM. They are secreted from the central hole of the salt glands (Fig. 2N). Flame photometry was performed for rapid and quantitative measurements of bulk ion concentrations and X-ray microanalysis (EDAX) for high-resolution spatial mapping of ions at the cellular level (Fig. 2T). The combination of these techniques provides a comprehensive understanding of how ion distribution and localization within plant tissues influence the response to salt stress. We determined the potassium (K), sodium (Na), and chloride (Cl) ion concentrations of leaf ash as measured by the flame photometric methods showed that 1 g of leave powder contains sodium (6.33%), chloride (13.3%), and potassium (1.16 %). In addition, X-ray

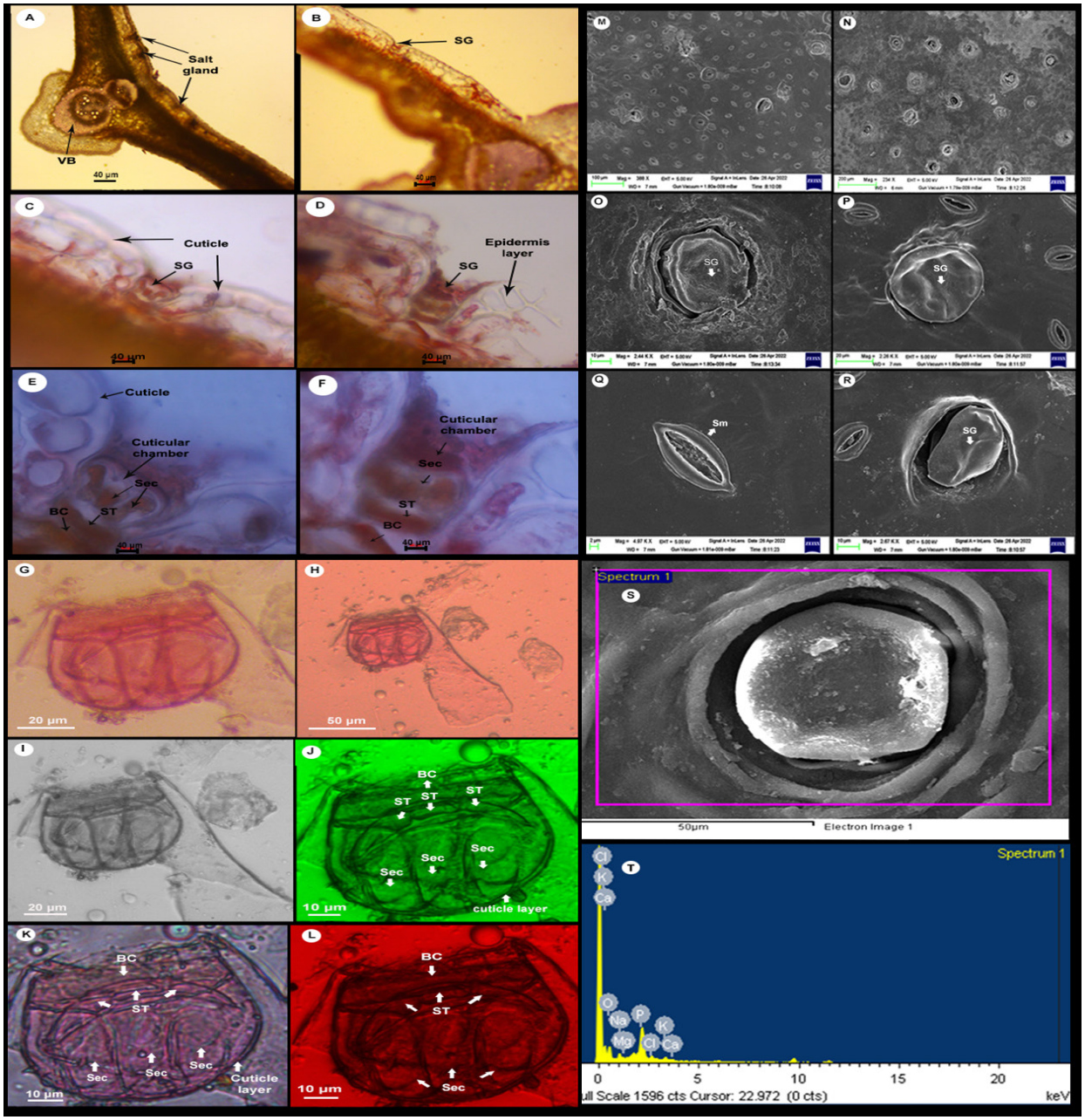

Fig. 2: Light microscopy images (Transverse section) (A-F). A- T.S. of *A. ilicifolius* isolateral leaf with vascular bundle; B- upper epidermis of *A. ilicifolius*; C- salt gland (multicellular, Asteride type); D-F- T.S. of Salt gland. (SG- salt gland, Sec- secretory cavity, ST- stalk cell, BC- basal cell, Epi- epidermal cell, VB- vascular bundle). Isolated gland images (Light microscopy) (G - L). salt gland, (SG- salt gland, Sec- secretory cavity, ST- Stalk cell, BC- Basal cell). SEM images (M- R). M- Leaf Abaxial surface; N- Adaxial surface; O- salt gland (adaxial); P- Secretory cavity (abaxial); Q- stomata; R- salt gland. (Sm- stomata, SG- salt gland, Sec- secretory cavity). SEM X-ray microanalysis (S-T). S- selected salt gland; T- Energy Dispersive Analysis of X-ray (EDAX) value.

microanalysis showed that the balls contained Ca, Mg, K, Cl, Na, O, and Si, which comprised 11.98, 4.98, 9.05, 8.59, 13.36, 51.99, and 0.05% of the total weight, respectively (Fig. 2T). Various studies have demonstrated that EDAX of the leaf surface of *Aeluropus littoralis* revealed that salt crystals are composed of sodium and chloride ions and other ions such as carbonate, calcium, chloride, magnesium, nitrate, phosphate, potassium, sodium, and sulfate. Lanthanum, rubidium, and silicon may also be involved. (Sakai, 1974; Thomson *et al.,* 1969; Thomson, 1975).

Conclusion

The salt glands (SGs) were successfully isolated from *A. ilicifolius* L. by describing the enzymatic methods. Detailed studies of the isolated gland ultrastructure revealed their supportive cells, such as secretory cells, basal cells, and stalk cells, which are responsible for salt secretion. This enables the efficient removal of excess salts, facilitating osmoregulation and maintaining ion balance within the plant. Autofluorescence imaging was used

to better visualize glandular structure without needing external staining. Salt glands in *A. ilicifolius* L. are sporadically scattered on both leaf epidermal surfaces under natural habitat, they excrete high levels of sodium, chloride and low levels of potassium ions. Excess salt may flow from the secretory cells via the stalk cell and basal cell. Quantification of accumulated inorganic ions of SGs revealed that the glands are mainly composed of sodium (Na^+), magnesium (Mg^{2+}), potassium (K^+), calcium (Ca^{2+}), chloride (Cl^-), phosphorus (PO_4), which facilitate osmotic regulation. Moreover, analysis of inorganic ion concentrations in the leaf salt glands was analyzed to gain insight into their role in enzyme activation, photosynthesis, nutrient transport and ion regulation.

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Author Contribution

All authors participated in (a) research design and conception or analysis and interpretation of the data; (b) article writing or critical revision for intellectual content; and (c) approval of the final version.

Conflict of Interest

Conflicts of interest do not exist. A similar manuscript has not yet been submitted, nor is it being reviewed by another journal. The authors have no financial interest in the manuscript discussed and do not have affiliations with any organizations.

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Ethical Approval

No applicable.

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