International Journal of Botany Studies
www.botanyjournals.com
ISSN: 2455-541X
Received: 14-03-2025, Accepted: 13-04-2025, Published: 29-04-2025
Volume 10, Issue 4, 2025, Page No. 58-63

Visualizing plant mitochondria

Janaki Subramanyan[1], Kumari Amya[2], Shreya Singh[2]
Department of Botany, Miranda House, University of Delhi, Delhi, India
[1] Former Professor, Department of Botany, Miranda House, University of Delhi, Delhi, India
[2]Students of B.Sc. Life Sciences II Year, Miranda House, University of Delhi, Delhi, India

Abstract
Mitochondria are difficult to observe because of the small size and lack of pigments. The size of mitochondria resembles that of bacteria. Moreover, plant cells have a cell wall which hinders with the rapid uptake of stains. In the present study the techniques of histological staining and light microscopy have been used to visualize and localize mitochondria. The smartphone camera has been used to take photographs, and the laptop has been used to suitably magnify the photographs by zooming to discern the fine details of cell structure. Supravital staining by Janus green B, and staining with methylene blue gave best results. Leaves of *Allium fistulosum* and *Crinum asiaticum* were the experimental materials chosen for preparing epidermal peels. Mitochondria could be observed in the cytoplasm and in the region around the nucleus in the epidermal cells. The guard cells of stomata showed abundant mitochondria. Our work is in agreement with the previous reports on mitochondria, and emphasizes the importance of the use of smartphones in teaching-learning to understand cell structure.

Keywords: Mitochondria, histological stains, Janus green B, *Allium fistulosum*, *Crinum asiaticum*, guard cells

Introduction

Mitochondria are ubiquitous, pleomorphic, double membrane-bound organelles found in eukaryotic cells. In animal and yeast cells, mitochondria are tubular-reticulate structures and several micrometers long (Jaipargas *et al* 2015) [11]. In green plants mitochondria are predominantly punctate in form with 0.2–1.5 μm punctae (Jaipargas *et al* 2015) [11]; or spherical or rod-shaped structures measuring 1–3 μm in length and about 0.5 μm in diameter and the chondriome (refers collectively to all mitochondria in a cell) of a plant cell may contain up to several hundred mitochondria (Møller *et al* 2021) [17]. The plant mitochondria are dynamic and vital organelles involved in respiration; photorespiration; β-oxidation of fatty acids; and synthesis of phospholipids, nucleotides and many amino acids (Jakobs *et al* 2020 [12], Logan 2006) [14]. Mitochondria remain as individual organelles as well as form networks which keep undergoing changes because of fusion and fission (Walker & Moraes 2022) [25]. The narrow diameter (200 to 700 nm) of the mitochondrial tubules is close to the diffraction limit of light microscopy, thereby, preventing clear visualization of mitochondria (Jakobs *et al* 2020) [12].

The small size resembling that of bacteria and lack of pigments make it difficult to observe mitochondria in cells. The presence of cell walls in plant cells also restricts the entry of stains, thereby, making it challenging to demonstrate the presence of mitochondria is plant cells under the light microscope. Janus green B, a basic dye, was introduced as a supravital stain for mitochondria by Leonor Michaelis in 1900. Plant mitochondria have been studied using Janus green B (Sorokin 1938, 1941) [22, 23] which is based on the activity of cytochrome *c* oxidase present in mitochondria (Lazarow & Cooperstein 1953 [13], Ruthman 1966) [19]. Avers & King (1960) [2] obtained mitochondrial counts using Janus green B in root tip meristem cells of four grass species. The fluorescent dyes, namely 3,3′-dihexyloxacarbocyanine iodide (Matzke & Matzke 1986) [15] and rhodamine 123 (Wu 1987) [26], have been used as vital stains to visualize mitochondria in living plant cells. Besides electron and fluorescence microscopy, the sophisticated techniques super-resolution microscopy which improves resolution and nanoscopy which overcomes the diffraction barrier have been used to study and observe mitochondria (Jakobs *et al* 2020) [12].

This investigation is an attempt to observe and localize mitochondria using the light microscope in undergraduate laboratories, taking photographs using the camera of a smartphone, and, thereafter, zooming the photographs to get a clear and magnified view to enable understanding and interpreting the cell inclusions. Leaves are sites of active metabolism; and the guard cells of stomata have a high rate of respiration. *Allium fistulosum* L. and *Crinum asiaticum* L. were chosen as the experimental materials for preparing leaf epidermal peels for the study. The supravital stain Janus green B (Lazarow & Cooperstein 1953 [13], Ruthman 1966 [19]), and the histological stains methylene blue (Prasad & Prasad 1975) [18] and rhodamine B (Bajracharya 1999) [3] have also been used to visualize and localize mitochondria under the light microscope. Additionally, commonly used stains, namely safranin, acetocarmine and iodine reagent, have been used to study the cell structure.

Materials and Methods

Fresh leaves of spring onion (*Allium fistulosum* L.) and giant/ grand crinum lily (*Crinum asiaticum*. L.) (both of family Amaryllidaceae) were used. Fresh uprooted plants of *A. fistulosum* were obtained from the local vegetable market and *C. asiaticum* leaves were collected from the botanical garden of the college.

Preparation of epidermal peels

The leaves of *A. fistulosum* are hollow and epidermal peels were prepared from the green portion of the leaves above the region where the leaves are clasped together. The lower epidermis of the leaves was used to prepare peels from *C. asiaticum.*

Use of different histological stains

Separate epidermal peels were used for each stain in the investigation. The leaf epidermal peels were stained with safranin (1 % in 50 % ethanol) for around three minutes; the stain was blotted and then the peels were rinsed with water to remove the excess stain. Thereafter, the peels were mounted in glycerine and observed under the light microscope. Leaf epidermal peels were placed in a drop of acetocarmine (2 % in 45 % acetic acid) taken on a microslide, gently warmed and then a coverslip was lowered. The preparation was observed under a light microscope. Iodine reagent (1 g iodine + 2 g potassium iodide dissolved in 300 mL distilled water) was also used as a stain to prepare leaf epidermal peel mounts. Leaf epidermal peels were treated for 30 minutes in rhodamine B (0.05 %, aqueous) taken in a cavity block with the lid. Epidermal peels were treated with methylene blue (0.2 %, aqueous) for around 3 minutes. The peels stained with rhodamine B or methylene blue were rinsed with water to remove the excess stain, mounted in glycerine and then observed under a light microscope. Although rhodamine B is a fluorescent dye, a light microscope which is available in all undergraduate teaching laboratories was used to observe the stained preparations.

Supravital staining using Janus green B

Epidermal peels were treated for 30 minutes with Janus green B (0.1 % in absolute ethanol) taken in a cavity block. To provide sufficient oxygen for the reaction to occur and to retain Janus green B in the coloured oxidized state the cavity block was covered with a beaker instead of the lid of the cavity block. Following staining the peels were rinsed with water and then mounted in glycerine and immediately observed.

Observation of the preparations

All the mounted preparations were observed under a compound microscope, using a 10x eyepiece lens and different magnifications of objective lens (10x, 40x and 100x). Photographs of the different preparations were captured using the camera of a smartphone. The maximum magnification that can be theoretically achieved using a 10x eyepiece, a 100x oil immersion objective and 10x smartphone camera lens would be 10,000x which is not practically feasible because of blurring of the image. Therefore, photographs were taken at combination of magnifications where sharp resolution of the image was obtained; the images were studied in a laptop and, thereafter, zoomed suitably, if necessary, to understand the fine details of the cell inclusions. Representative photographs have been used to discuss the results. A stage micrometer was used to calculate the magnifications of the photographs.

Results and Discussion

The results obtained using the common stains are discussed first in order to provide an understanding of the cell structure of the epidermis of the two taxa studied. The leaf epidermal cells are elongated and polygonal; the cells of *A. fistulosum* were long and narrow compared to the epidermal cells of *C. asiaticum*. Both the materials had anomocytic stomata (Fig 1 A, B, E). In the safranin-stained preparation of *A. fistulosum* (Fig 1 C) the cell walls and nuclei were clear; safranin stained the cutinized cell walls and chromatin (Prasad & Prasad 1975) [18]. Acetocarmine, a nuclear stain (Prasad & Prasad 1975) [18], stained the nucleus red; the nuclei of the bean-shaped guard cells are also clear (Fig 1 D). The stomata are closed and the inner thick cell walls of the guard cells are distinct (Fig 1 A, C, D). In the iodine reagent-stained preparation of *A. fistulosum* the nuclei in all cells (please see arrow in Fig 1 B) and the cell inclusions turned yellow simply because iodine was retained in these organelles. However, the chloroplasts in guard cells did not turn blue-black (Fig 1 B). This showed that the guard cell chloroplasts lacked starch and hence no blue-black starch-iodine complex could be formed (Gupta 1996) [8]. Our observation is in agreement with the fact that starch is absent in the guard cells of *A. cepa* (Heath 1952 [10], Schnabl & Ziegler 1977) [20]. It has been reported that guard cells of onion contain soluble fructans which may provide the suitable osmoticum for stomatal opening, and sucrose uptake from the apoplast may be an additional source of carbohydrate (Amodeo *et al* 1996) [1]. Contrastingly, the guard cells of *C. asiaticum* had several globoid chloroplasts containing starch which turned blue-black on staining with iodine reagent (Fig 1 E). The nuclei in the cells of *C. asiaticum* also retained iodine and appeared pale yellow (Fig 1 E).

Rhodamine B stained the nuclei, chloroplasts and mitochondria purple in *C. asiaticum* (Fig 1 F). Numerous globoid chloroplasts were present in the guard cells. The particulate inclusions in the cell, especially concentrated around the faintly stained nucleus are the mitochondria (see also inset of Fig I F). It is quite likely that leucoplasts also got stained with rhodamine B. The mitochondria in the guard cells cannot be differentiated as distinct entities because of the chloroplasts. However, the guard cells presumably contain abundant mitochondria.

When methylene blue was used as the stain, the mitochondria were observed in the epidermal cells of *A. fistulosum* (Fig 2 A). The mitochondria were observed as fine blue particles of variable shape and concentrated in the epidermal cells in regions adjacent to the guard cells and around the nucleus, and in the guard cells (Fig 2 A, B). Interestingly, because of the abundance of mitochondria, the guard cells appear blue. Methylene blue stained the nucleus as well. It is quite likely that some of the blue particles are leucoplasts. Methylene blue is routinely used as a stain for bacteria and yeasts (Prasad & Prasad 1975) [18].

Janus green B stained the mitochondria blue in *A. fistulosum* epidermal cells (Fig 2 C, D). The mitochondria are observed as blue particles of variable shape. The chloroplasts in the guard cells are not visible (Fig 2 C, D). Numerous mitochondria are present around the nucleus of the epidermal cell on the left of the stomate and in the epidermal cell above the stomate (Fig 2 C) and in the guard cells (Fig 2 D). Squamous epithelial cells of the buccal cavity also show mitochondria mainly around the nucleus (Gupta *et al* 2018) [9]. The epidermal cells of *C. asiaticum* also showed the presence of mitochondria which have stained greenish blue (Fig 2 F). The chloroplasts in the guard cells are large, round, yellow and not distinct in all cells because of the innumerable mitochondria (Fig 2 E, F). In some cells it is evident that the mitochondria have clustered around the nucleus (Fig 2 F). Comparison of Fig 1 E, F and Fig 2 E with Fig 2 F clearly shows that the size of chloroplasts in the guard cells is larger than mitochondria and that the chloroplasts do not get stained with Janus green B. The mitochondria in the guard cells of *C. asiaticum* are numerous and greenish blue amidst the chloroplasts which are not stained (Fig 2 F). In both the experimental materials Janus green B also stained the nucleus. The mitochondria are able to retain the Janus green B in the oxidized state because of the activity of the enzyme cytochrome *c* oxidase, whereas the remaining part of the cell lacks cytochrome *c* oxidase and Janus green B gets reduced to its leucobase (Lazarow & Cooperstein 1953) [13]. Janus green B selectively stains the mitochondria and the staining reaction indicates that the cell is viable and mitochondria are functional. Cytochrome *c* oxidase is a large transmembrane protein bound to the plasma membrane in aerobic bacteria, and to the inner mitochondrial membrane in eukaryotes. The enzyme, also referred to as Complex IV in the mitochondrial electron transport chain (Siedow & Day 2000) [21], is a key enzyme in aerobic metabolism (Castresana *et al* 1994) [4]. However, with the passage of time when oxygen gets cut off because of the presence of the coverslip on the epidermal peel, the mitochondria will start getting decolorized. In both methylene blue- and Janus green B-stained preparations in both the taxa studied the guard cells were filled with mitochondria (Fig 2 B, D, F). The abundance of mitochondria in epidermal cells at sites facing the guard cells (Fig 2 B, C), and in guard cells (Fig 2 B, D, F) is explained on the basis of the fact that the guard cells are actively involved in stomatal movements which require energy; the mitochondria help in providing the ATP required for the active transport of H^+ ions from the guard cells and for the secondary active transport of K^+ and Cl^- ions into the guard cells. It is known that guard cells contain a very large number of mitochondria, and show high expression of H^+-ATPases and ATPases; the activity of mitochondrial enzymes and the respiratory rate in guard cells are higher than in mesophyll cells (Daloso *et al* 2017) [6]. In fact, guard cells have more mitochondria than chloroplasts (Gahir *et al* 2024) [7].

Our observation of mitochondria clustering around the nucleus (please see arrows in Fig 2 B, C, F) corroborates the work in previous reports. In tips of aerial roots of *Chlorophytum capense* using electron microscopy Miguel (1963) showed the formation of invaginations in the nucleus where mitochondria accumulated and many mitochondria were attached to the nuclear membrane. It was suggested that the attachment sites were sites where some substances were being exchanged or absorbed by the nucleus (Miguel 1963) [16]. Using 3-D reconstruction of fluorescence and the technique of FRAP (Fluorescence Recovery after Photobleaching) in different types of animal cells Collins *et al* (2002) [5] observed an aggregation of mitochondria in perinuclear positions. According to the study mitochondria exist as separate entities and were not connected, and the morphology depends on the balance of fusion and fission processes, and probably there is no single morphology or functional status even for an individual cell. Electron microscopic studies have shown that clusters of mitochondria are present around the nucleus in the basal epithelial cells and superficial epithelial cells in the epidermis of larval zebrafish skin, and nucleus-mitochondria membrane contact sites were formed as a process preceding mitochondria destruction. The mitochondria are engulfed by extensions of the nuclear envelope as the epithelial cells mature and stratify (Tracey-White & Hayes 2024) [24]. Mitochondria interact with other organelles through signalling pathways and contact sites; mitochondria depend on the nuclear genes for the majority of the proteins required and the relationship between the mitochondria and the nucleus is crucial for the cell's survival (Walker & Moraes 2022) [25].

Conclusions

Mitochondria could be observed in *A. fistulosum* and *C. asiaticum* leaf epidermal peels. Staining with methylene blue and Janus green B showed that mitochondria were abundant in the guard cells and in epidermal cells at regions adjacent to the guard cells. Mitochondria were present in large numbers around the nucleus. The study shows that for visualizing plant mitochondria the technique of light microscopy can be aided with photography using the smartphone, and magnifying the photographs by zooming in a laptop.

Acknowledgements

We sincerely thank Professor Bijayalaxmi Nanda, Principal, Miranda House, University of Delhi, Delhi 110007, for providing the facilities, and Dr Shruti Kasana, Assistant Professor, Department of Botany, University of Delhi, Delhi 110007, for identification of spring onion. The laboratory staff of the Department of Botany, especially Mr Umesh Chandra Joshi and Mr Dinesh Giri, were very supportive and we thank them.

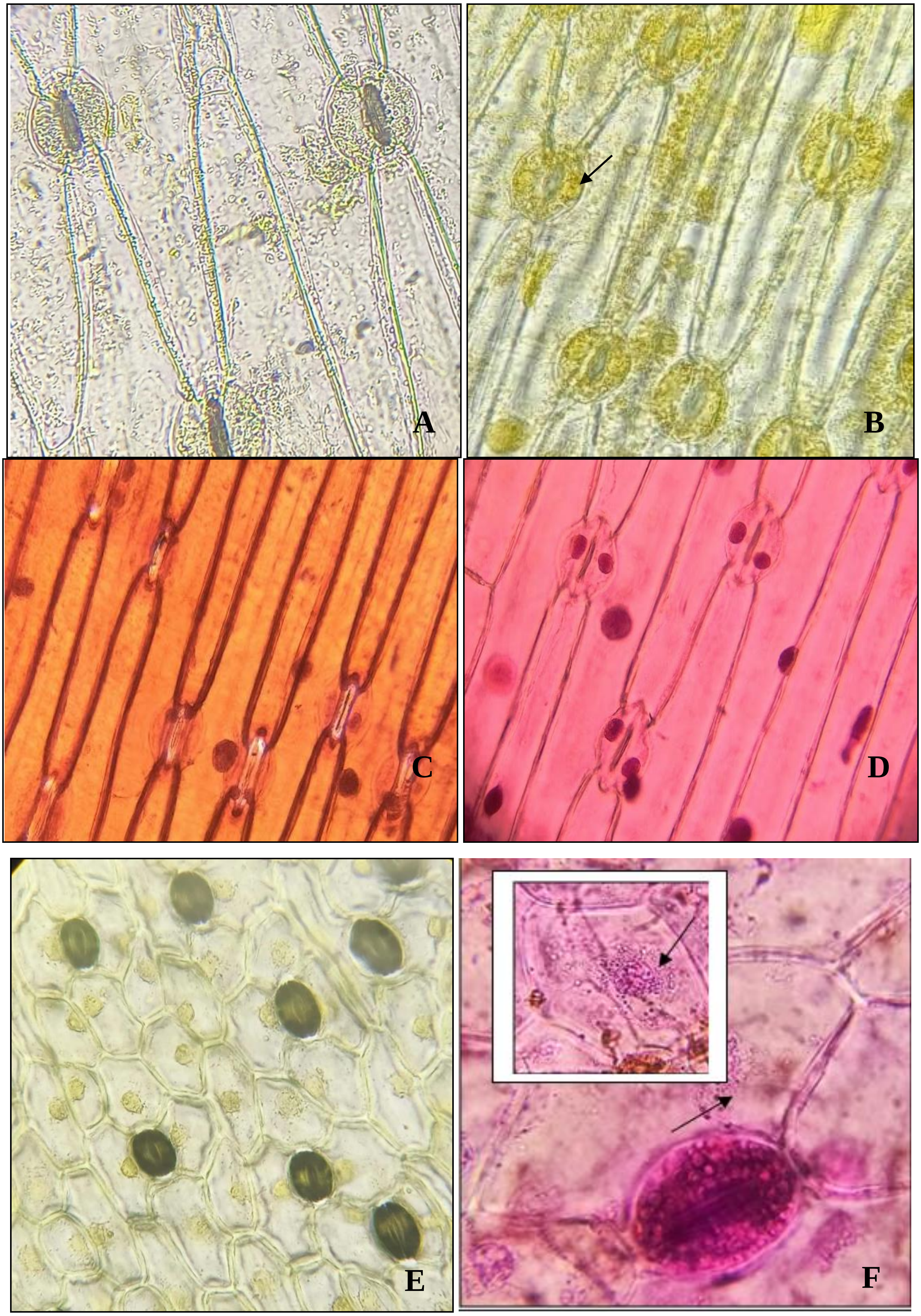

Fig 1: A-D: Peel mounts of the leaf epidermis of *A. fistulosum*. A. In water (1410x). B. Stained with iodine reagent (1128x). The arrow points to the nucleus in the guard cell. C. Stained with safranin (1034x); D. Stained with acetocarmine (1128x). E, F: Peel mounts of the lower epidermis of the leaf of *C. asiaticum*. E. Stained with iodine reagent (846x). F. Stained with rhodamine B (2726x). The inset shows a cell with its nucleus surrounded by mitochondria. The arrows point to the mitochondria around the nucleus.

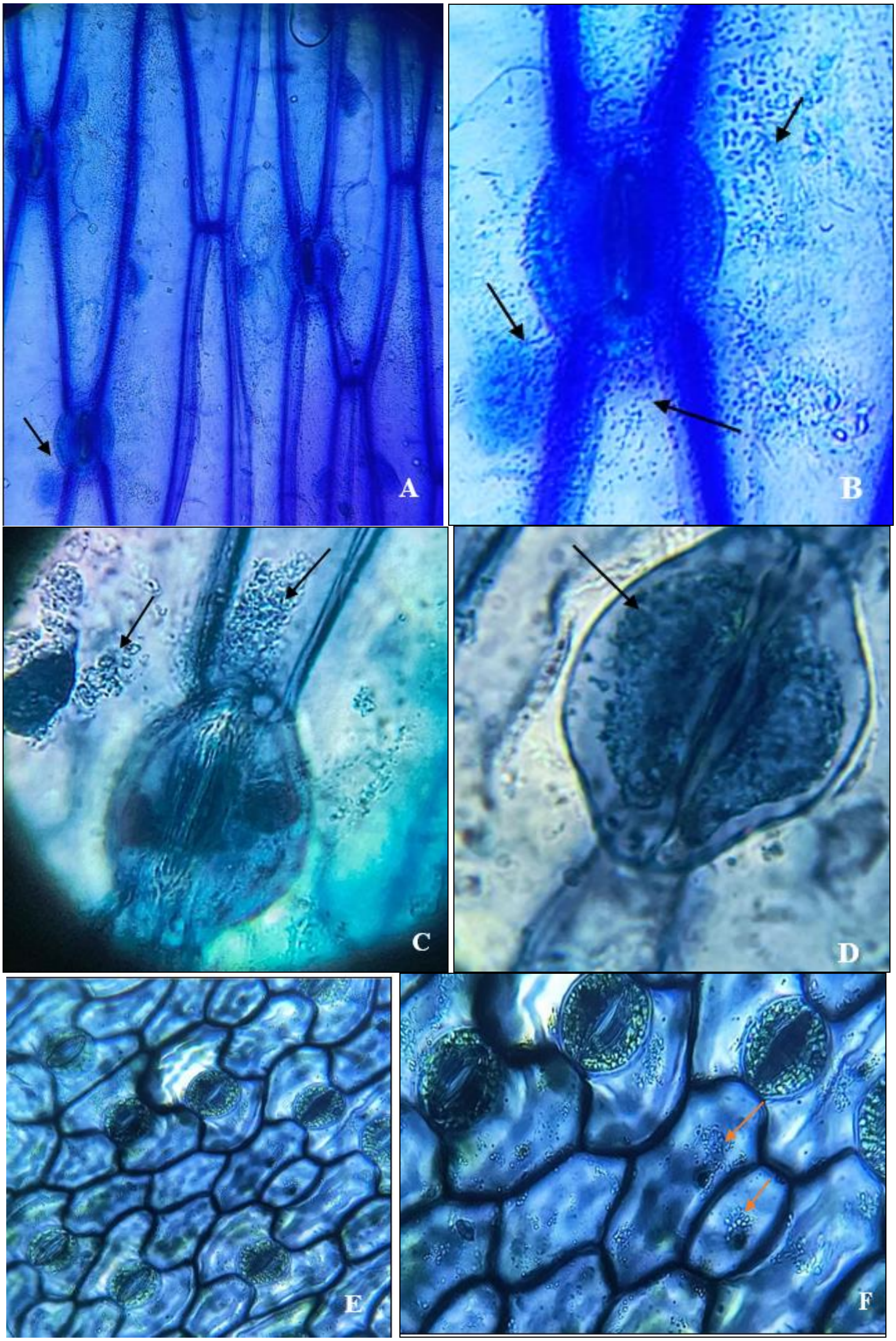

Fig 2: A-D: Peel mounts of the leaf epidermis of *A. fistulosum*. A, B. Stained with methylene blue. The stomate in A (arrow) has been magnified in B. The arrow on the left in B points to the nucleus surrounded by mitochondria; the arrows on the right show the clustering of mitochondria in epidermal cells in regions adjacent to the guard cells. A. 1034x; B. 3384x. C, D. Stained with Janus green B, arrows point to mitochondria; C: 3760x; D: 5452x. E, F: Peel mount of the lower epidermis of the leaf of *C. asiaticum* stained with Janus green B; E. 752x; F. 1504x. The arrows in F point to the greenish blue mitochondria.

References

1. Amodeo G, Talbott LD, Zeiger E. Use of potassium and sucrose by onion guard cells during a daily cycle of osmoregulation. Plant Cell Physiology,1996:37(5):575-579.
2. Avers CJ, King EE. Histochemical evidence of intracellular enzymatic heterogeneity of plant mitochondria. American Journal of Botany,1960:47(3):220-225. https://doi.org/10.1002/j.1537-2197.1960.tb07117.x
3. Bajracharya D. Experiments in Plant Physiology, New Delhi. Narosa Publishing House,1999, 2-3.
4. Castresana J, Lübben M, Saraste M, Higgins DG. Evolution of cytochrome oxidase, an enzyme older than atmospheric oxygen. The EMBO Journal,1994:13(11):2516-2525.
5. Collins TJ, Berridge MJ, Lipp P, Bootman MD. Mitochondria are morphologically and functionally heterogeneous within cells. The EMBO Journal,2002:21(7):1616–1627. https://doi.org/10.1093/emboj/21.7.1616
6. Daloso DM, Medeiros DB, dos Anjos L, Yoshida, T, Araújo WL, Fernie AR. Metabolism within the specialized guard cells of plants. New Phytologist,2017:216(4):1018-1033.
7. Gahir S, Bharath P, Saini D, Padmaja G, Raghavendra AS. Role of mitochondria and chloroplasts during stomatal closure: subcellular location of superoxide and H_2O_2 production in guard cells of *Arabidopsis thaliana*. Journal of Biosciences,2024:49(2):44. https://doi.org/10.1007/s12038-023-00418-3
8. Gupta K. Carbohydrates. In: Sawhney SK, Singh R (ed) Introductory Practical Biochemistry, New Delhi. Narosa Publishing House Pvt. Ltd., 1996, 17-18.
9. Gupta R, Makhija S, Toteja R. 2018. Cell Biology Practical Manual, New Delhi. Prestige Publishers, 2018, 110-112.
10. Heath OVS. Studies in stomatal behaviour. II. The role of starch in the light response of stomata. Part 2. The light response of stomata of *Allium cepa* L., together with some preliminary observations on the temperature. New Phytologist,1952:51:30-47.
11. Jaipargas EA, Barton KA, Mathur N, Mathur J. Mitochondrial pleomorphy in plant cells is driven by contiguous ER dynamics. Frontiers in Plant Science, 2015:6: Article 783. doi:10.3389/fpls.2015.00783. PMID:26442089; PMCID: PMC4585081.
12. Jakobs S, Stephan T, Ilgen P, Brüser C. Light microscopy of mitochondria at the nanoscale. Annual Review of Biophysics,2020:49:289-308. https://doi.org/10.1146/annurev-biophys-121219-081550
13. Lazarow A, Cooperstein SJ. Studies on the enzymatic basis for the Janus green B staining reaction. Journal of Histochemistry & Cytochemistry,1953:1(4):234-241. doi:10.1177/1.4.234. PMID:13069688.
14. Logan DC. Plant mitochondrial dynamics. Biochimica et Biophysica Acta (BBA) - Molecular Cell Research,2006:1763(5–6):430-441. https://doi.org/10.1016/j.bbamcr.2006.01.003
15. Matzke MA, Matzke AJM. Visualization of mitochondria and nuclei in living plant cells by the use of a potential-sensitive fluorescent dye. Plant, Cell & Environment,1986:9:73-77.
16. Miguel M. Electron microscope study of the relationship between the nucleus and mitochondria in *Chlorophytum capense* (L.) Kuntze. Cytologia,1963:28(4):409-416.
17. Møller IM, Rasmusson AG, Aken OV. Plant mitochondria – past, present and future. The Plant Journal,2021:108(4):912-959. https://doi.org/10.1111/tpj.15495
18. Prasad MK, Prasad MK. Outlines of Microtechnique, Delhi. Emkay Publications, 1975, 37-45.
19. Ruthman A. Methods in Cell Research, Ithaca, New York. Cornell University Press,1966, 182.
20. Schnabl H, Ziegler H. The mechanism of stomatal movement in *Allium cepa* L. Planta,1977:136(1):37-43. DOI:10.1007/BF00387922
21. Siedow JN, Day DA. Respiration and photorespiration. In: Buchanan BB, Gruissen W, Jones RL (ed) Biochemistry & Molecular Biology of Plants, New Delhi. IK International Pvt. Ltd.,2000, 689.
22. Sorokin H. Mitochondria and plastids in living cells of *Allium cepa*. American Journal of Botany,1938:25(1):28-33.
23. Sorokin H. The distinction between mitochondria and plastids in living epidermal cells. American Journal of Botany,1941:28:476-485.
24. Tracey-White D, Hayes MJ. Nucleus-mitochondria contact sites are associated with asynthetic fission in zebrafish skin. Contact: The Journal of Inter-Organelle Communication,2024:7:25152564241239445. doi:10.1177/25152564241239445. PMID:38524404; PMCID: PMC10958491.
25. Walker BR, Moraes CT. Nuclear-mitochondrial interactions. Biomolecules,2022:12(3):427. https://doi.org/10.3390/biom12030427
26. Wu FS. Localization of mitochondria in plant cells by vital staining with rhodamine 123. Planta,1987:171:346–357. https://doi.org/10.1007/BF00398680