



Deciphering Regulatory Role of Melatonin in Foliar Nastic Movements of *Portulaca oleracea*: Visual Insights from Histology and Anatomy

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Abstract

Plant nastic movements exhibit unique behavioural patterns that synchronize with external cues. Given that the foliar nastic motions of *Portulaca* species are solely circadian, it is intriguing to investigate whether and how melatonin governs these movements. Analysis of histological traits concurrent with anatomical traits such as stomatal behaviour provides visual data on the plant species' gnosophysiology, offering clues and validation of the influence of multiple external stimuli on hydraulic forces that in turn alter turgor pressure. The current study aims to elucidate how exogenous melatonin modulates foliar nastic movements in *Portulaca oleracea*. Our findings indicate that melatonin functions as an intracellular hydraulic flux controller, influencing idioblast and crystal densities, as well as stomatal behaviour. Timepoint studies at specific Zeitgebers reveal that abiotic variables such as light and temperature can influence the endogenous melatonin concentration of *P. oleracea*. Therefore, this hormone potentially serves as an additional internal regulator of turgor pressure, influenced by both light and temperature. Thus, melatonin plays a crucial role in shaping the characteristic foliar nastic movements observed in *P. oleracea*, inherently tied to the circadian rhythm. Further investigation into idioblast and crystal torques, along with their angular momentum, is warranted to calculate the hydraulic forces at work in the leaf lamina. Thus the study underscores the multifaceted role of melatonin in navigating nastic movement processes through turgor pressure alterations brought about by intracellular depositions. The study in future could unravel melatonin's pleiotropic actions and the underlying mechanisms of foliar idiosyncratic nastic motions in other plant species as well. Moreover, both histology and anatomy play vital roles in the present study as it has provided visual evidence of the underlying mechanisms of foliar nastic movements in *P. oleracea*.

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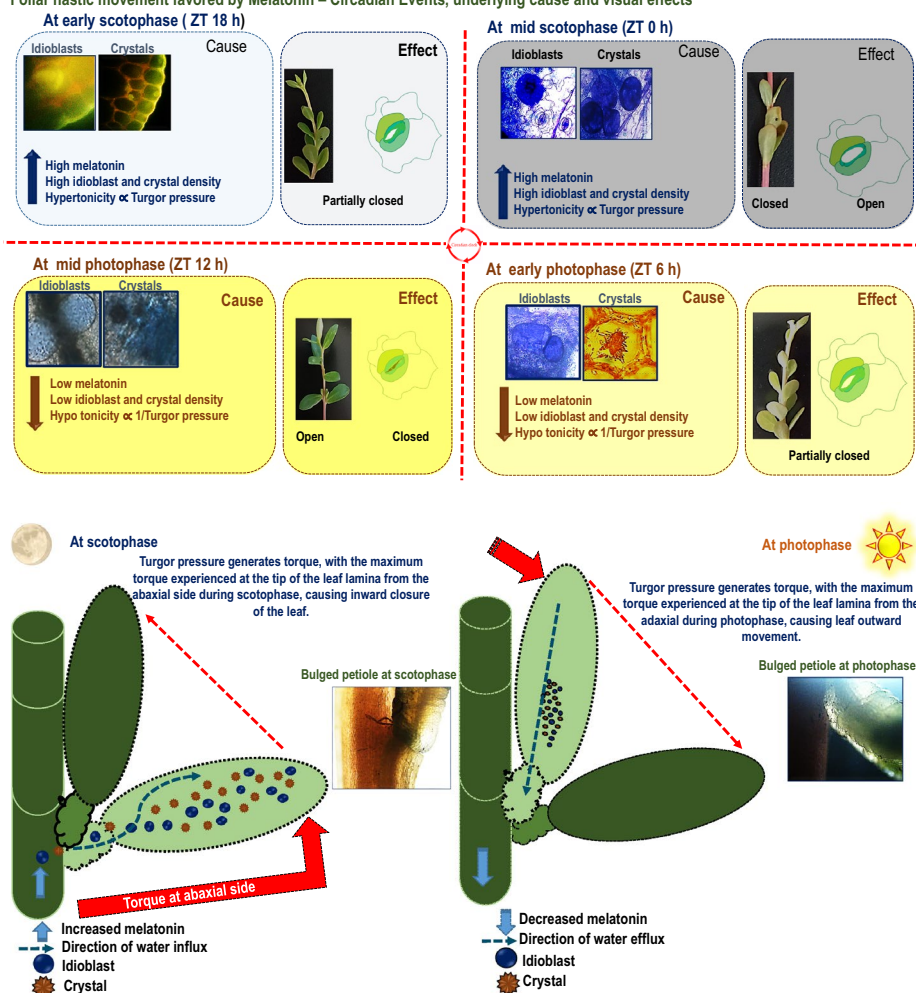
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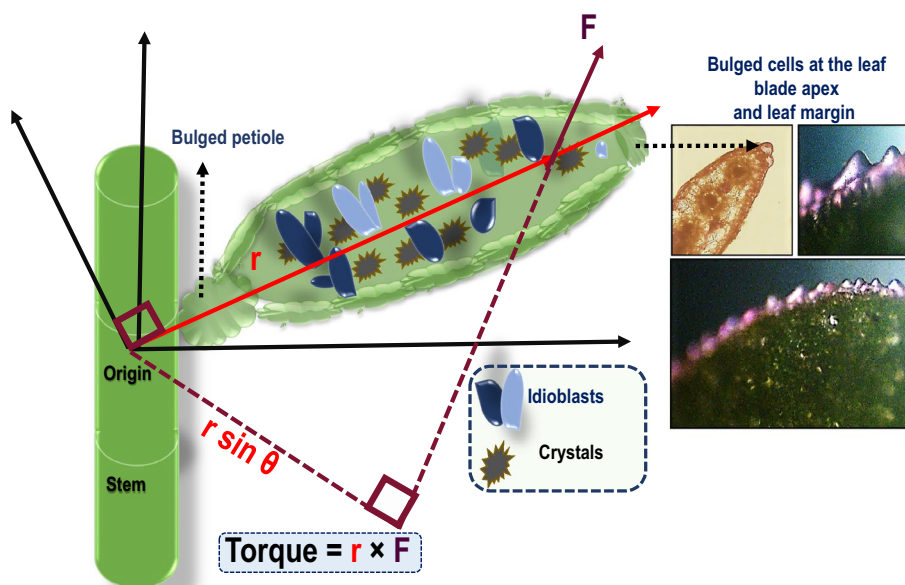
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Graphical Abstract

Foliar nastic movement favored by Melatonin – Circadian Events, underlying cause and visual effects



Rotational force in action rendering foliar nastic movement



Keywords Anatomy · Circadian · Gnosophysiology · Histology · Melatonin · Nastic movements · *Portulaca oleracea* · Zeitgeber

Introduction

Behavioural ecology aims to comprehend how organisms evaluate and react to environmental stimuli. They analyse environmental cues and respond to them in a manner akin to animals. Plant gnosophysiology refers to the branch of life science that deals with its cognition and response to stimuli. Most biologists refer to this as plasticity. Occasionally, researchers have openly borrowed theories and models from animal behavioural ecology to formulate concepts related to plant plasticity (Brooker et al. 2022; Jessup et al. 2022; Mu et al. 2022). Plant cells are equipped with systems enabling them to encode and decode information within their cells and tissues, facilitating adaptive plasticity in response to environmental signals. Whilst plant behaviours are manifested through movement, their sessile nature means that most movements are intricately linked to growth (de Bang et al. 2021; Sousa-Baena et al. 2021). The motions of growing plants are generally categorized into nastic movements, which are non-directional and internally driven, and tropic movements, which are directional and environmentally driven. Nastic movements are thus in synchrony with specific plant behavioural patterns.

Plants are continually subject to varying internal and external mechanical stimuli, which can range from stem flexion caused by tree weight and wind drag to stem nutation, cell development variability, and nanoscale wall component deformation (Mouliia et al. 2021). The responses of the plants appear conflicted. The repeatability of plant morphogenesis and development might be put at risk by variations, which can cause mechanical risks (e.g., growth fissures and windbreaks resulting from mechanical shear between heterogeneously growing cells). To rebuild their configuration (from the cellular to the tissue level); propel morphogenesis; and regulate their fate, plants appear to increase and even induce some movements where the cause of the stimuli is unknown (Soukup et al. 2021). Additionally, oscillating behaviours in tissues may result from purposefully enhancing mechanical cue fluctuations. Leaf nastic motions such as proprioception may encourage organ flattening (Geldhof et al. 2021). According to studies, fluctuation-enriched proprioception enables plant tissues to perceive other organisms and adapt to changing internal or external settings with flexible outputs (Mouliia et al. 2021).

Plants use different water acquisition methods and thus directly or indirectly obtain environmental resources. The anatomical environment uses specific visual cues to support spatiotemporal water distribution, which is circadian in species. The combination of ion action potentials (similar

animal neural network) encodes information through rapid changes in turgor pressure and cellular structural elasticity, causing nastic movement (Medina et al. 2021). Calcium oxalate (CaOx) and carbonate (CaCO₃) crystals are found in all taxa of autotrophic organisms, ranging from microalgae to angiosperms and the gigantic gymnosperms (Malekhosseini et al. 2022). CaOx crystal production in plants serves several vital tasks, including high-capacity calcium (Ca) control and herbivory defence (Karabourniotis et al. 2020). Thus the biomineralization phenomenon, such as crystal formation, is not a random physico-chemical manifestation of endogenously generated oxalic acid and environmentally obtained calcium, as proven by ultrastructural and developmental investigations (Cerritos-Castro et al. 2022). Crystals appear in variant structures and sizes. The consistency of crystal phenotype within species, cell specialisation, and the extraordinary synchronization of crystal growth and cell enlargement all point to the genetic regulation of CaOx production (Zhang et al. 2019). Idioblasts are specific parenchyma cells encountered either as high or low in tissues. They function as floating storage cells and sometimes are seen to differentiate into laticifers (Uzaki et al. 2022). They are always found close to vascular tissues. They are also found to be pathogenesis-related metabolites and sometimes were found to act as calcium stores in some instances. They are also connected to cellular differentiation and cell division. In *Arabidopsis* leaves, the idioblasts often occur contiguously, indicating that additional cell division events occur after idioblast differentiation (Shimada et al. 2018). This is similar to the differentiation of stomatal myrosin cells. The toughness of a plant cell in response to an external force is influenced by the pliability of the cell wall and turgor pressure, and cell geometry, which affect cell wall pressure. Thus idioblasts and crystals seem to affect turgor pressure and stomatal behavioural patterns. These visual inferential determinants, such as idioblasts and crystals, aid in decoding the foliar nastic movements. The examination of morphological traits, such as leaf movement behaviour, anatomical features such as stomatal index, and histological properties including idioblasts and crystal densities, provides valuable insights into the impact of pleiotropic external stimuli on plant physiology and adaptation.

Melatonin is regarded to be a potential plant growth regulator, capable of increasing plant growth and tolerance to a variety of abiotic stresses (Park et al. 2019; Wang et al. 2022). Melatonin injection improved stomatal characteristics such as length, width, area, and the number of pores under drought stress conditions (Sun et al. 2022), thus maintaining ionic homeostasis (Talaat and Shawky 2022). Melatonin at

300 μM maintained photosynthetic capability in the absence of water by improving carboxylation efficiency and chlorophyll concentration (Campos et al. 2019). Since melatonin has circadian functions that alleviate stress in the plant (Chung and Deng 2020; Talaat and Shawky 2022), it might also have a role in nastic movements. The involvement of melatonin in controlling plant nastic movements remains an understudied aspect of plant physiology, lacking substantial empirical evidence. This gap in understanding underscores the need for a rigorous investigation into melatonin's potential regulatory role in this intricate process (Huang et al. 2024; Kumar et al. 2024; Sandoval et al. 2024).

Given melatonin's established role in regulating circadian rhythms in plants, it can be hypothesized that melatonin may modulate the circadian-driven foliar nastic movements observed in *P. oleracea*. Building upon melatonin's known influence on turgor pressure regulation in plant cells, it can be proposed that melatonin could directly impact the turgor-driven mechanisms underlying foliar nastic movements. Hence, the current investigation seeks to elucidate the intricate mechanisms underlying the influence of exogenous melatonin on the foliar nastic motions observed in *P. oleracea* (Fig. 1a–c). Despite the well-established circadian nature of these movements, the precise regulatory factors remain elusive. The present study endeavours to fill this gap by exploring the potential role of melatonin in orchestrating these rhythmic responses. Leveraging visual indicators, the present study aims to unravel the primary drivers behind the foliar nastic movements exhibited by *P. oleracea*, offering novel insights into the underlying physiological processes.

Materials and Methods

Plant Material, Growth Conditions, and Sample Size

The study comprised vegetatively propagated *Portulaca oleracea* (pink flower variety), which were cultivated in 80 pots filled with autoclaved, solarized, and pest-free soil, periodically cleared of weeds. Each pot was planted with five 10 cm-long stem-cuttings of *P. oleracea*. The life cycle and duration to reach the reproductive phase were meticulously documented. The plants were conditioned with a regular photoperiod of 16 h light (250–300 $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$)/8 h darkness at 30 °C in a growth chamber. After 75 days of conditioning, the plants were subjected to various treatments. Eighty plants were divided into 4 subgroups of 20 each. One group served as the control group, receiving no melatonin treatment, whilst the other three groups received melatonin dissolved in Hoagland's solution at three different concentrations (10, 50 and 100 μM). Melatonin, the neurohormone, was dissolved in Hoagland's solution, a well-established nutrient solution commonly utilized in

plant biology and agriculture for hydroponic cultivation (Hoagland and Arnon 1950). Therefore, control plants were treated with Hoagland's solution lacking melatonin.

Endogenous Melatonin Quantification in Whole Plants

The intrinsic melatonin concentration of the whole plant was measured at specific Zeitgeber Time (ZT 0 h, ZT 6 h, ZT 12 h, and ZT 18 h). 1 g of plant tissue collected at specific time points was subjected to a cold extraction procedure in the dark with liquid nitrogen in the prechilled mortar and pestle. The powder was extracted with 70% chilled ethanol. The samples were cold centrifuged for 15 min at 4000 rpm at a temperature of 4 °C. The supernatant was subjected to lyophilization. The lyophilized powder was redissolved in 100 μL of 70% ethanol and quantified using RP-HPLC with a fluorescent detector. The chromatographic system (Waters® Alliance e2695 Separations Module) was furnished with an autosampler, degasser and fluorescence detector (Waters® 2475 Multi λ Fluorescence detector). The stationary phase C18 reversed-phase column (250 $\times$ 4.6 mm, 5 μm) was equipped with a C8 pre-column. Before use, the mobile phase was filtered through a Phenomenex membrane filter (nylon, 47 mm membrane, 0.20 μm). An ultrasonic bath was used for degassing the solvents. The flow rate was 0.9 mL min^{-1} , and the injection volume was 40 μL . Fluorescence intensity was monitored at 352 nm, whilst excitation was detected at 298 nm. Isocratic elution mode via RP-HPLC facilitated an optimal separation of metabolites. Acetonitrile (ACN)—eluent 'A' and 0.001% Trifluoroacetic acid (TFA) in Milli-Q water—eluent 'B' in 65:35 (v/v) ratio comprised the mobile phase solvents. Chromatographic separation was achieved at a pressure of 1200–1300 psi set at 25 °C. The sample temperature was maintained at 4 °C. Samples viz., blank, melatonin standard, and test were injected into the RP-HPLC, and 7 min was fixed as the run time. Melatonin standard was procured from Sisco Research Laboratories Pvt Ltd, and the pure standard was prepared using 99.8% HPLC grade ethanol at a concentration of 1 mg mL^{-1} . Every solution of diluted standards was prepared fresh 30 min before use (for not more than 1 h in the dark) in amber-coloured tubes kept in ice to evade light/temperature-mediated degradation.

Exogenous Melatonin Administration

The control group comprised vegetatively propagated *P. oleracea* plants with 10 cm-long twigs, which were irrigated with Hoagland's solution. Hoagland's solution containing melatonin was utilized for irrigating the test plant group, whilst the control set received irrigation with Hoagland's solution devoid of melatonin. Melatonin spray

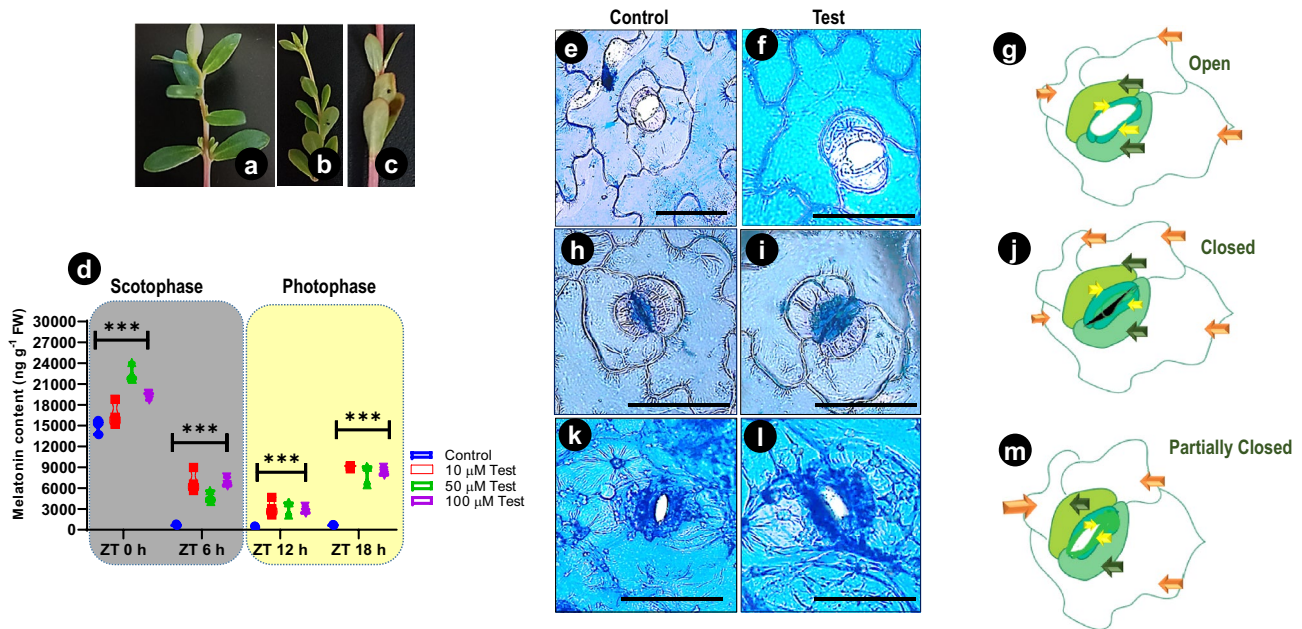


Fig. 1 Foliar nastic movements in *Portulaca oleracea* showing **a** fully open **b** partially closed and **c** fully closed leaves. **d** represents innate melatonin content at scotophase (ZT 0 h & ZT 6 h) and photophase (ZT 12 h & ZT 18 h) at specific Zeitgeber Time points in the Control group (plants without melatonin supplementation) and Test group (plants sprayed with melatonin in concentrations 10, 50, 100 μM).

Stomatal features in Control **e**, **h**, and **k** and Test **f**, **i**, **l**, **g** represents open stomata **j** represents closed stomata and **m** represents partially closed stomata. Yellow arrows represent guard cells, green arrows represent subsidiary cells and orange arrows represent epidermal cells. The type of stomata in *P. oleracea* is paracytic in nature. Scale bar = 100 μm

was administered to the treatment groups twice daily at ZT 6 h and ZT 18 h, with three different concentrations (10 μM, 50 μM and 100 μM). Sampling was conducted 30 days after the initiation of treatment, at specific time points on the same day. Each experimental group consisted of 20 replicates. Endogenous melatonin levels were analyzed in test samples following a 6-h window period after melatonin administration, and these levels were subsequently compared to those observed in the control group. The experimental design adopted was a randomized controlled trial (RCT) with multiple treatment groups. Specifically, it involved a controlled comparison between different groups (control group receiving no melatonin and test groups receiving melatonin at different concentrations) with random allocation of subjects (plants) to each group. This design allows for the assessment of the effects of the independent variable (melatonin concentration) on the dependent variable (endogenous melatonin levels) whilst controlling for potential confounding factors.

Sampling for Studying Stomatal Behaviour

Leaves from both the control and test sets of the species were randomly sampled 30 days after the melatonin treatment, specifically from plants that had reached reproductive age (75 days), encompassing both young and mature stages. Leaves were sealed in ziplock plastic bags, transported to

the laboratory, and fixed before morphological and anatomical analyses. Leaf length, thickness (width), and area were determined with ImageJ (National Institute of Health (NIH), Bethesda, Maryland). Leaf length divided by leaf width was used as an index of leaf lamina shape (low value = ovoid). The abaxial surface of three randomly selected leaves from the harvested group was painted (between two central lateral veins) with transparent nail polish. Peels from both the adaxial and abaxial surfaces were examined. On graph paper, the leaf was placed, and its dimensions were ascertained. Young leaves had to be at least 1 cm long, whilst mature leaves were 3 cm long. Mature leaves were always chosen from the second node from the stem's base, whilst young leaves were always selected from the second node from the tip. Stomatal indexing was determined for each leaf and then averaged over control vs test samples at four time points and three different concentrations. The control and test groups were compared, but there was no random assignment of treatments to groups. Instead, treatments were applied to different groups in a controlled manner. The experimentation entailed elements of randomization in the selection of leaves for analysis. Leaves were randomly sampled from both control and test sets of plants. Additionally, the use of randomization in selecting leaves helped reduce bias and ensure representative sampling. Two-sample groups, the treated and the control

were used to compare the results using analysis of covariance (ANOVA).

To calculate the stomatal density, all the stomata in the field of vision were counted in each image ($0.36 \text{ mm} \times 0.29 \text{ mm} = 0.105 \text{ mm}^2$). To measure stomatal size, five more photos were taken from the same epidermal peel (40 objective lenses, the field of view = $0.21 \text{ mm} \times 0.16 \text{ mm} = 0.034 \text{ mm}^2$). Using Image J software, the total number of pores in the field of view and the length of each stomatal pore were calculated. The stomatal index was calculated using the formula; $\text{Stomatal index} = \frac{\text{Stomatal density}}{\text{Stomatal density} + \text{Epidermal cells}}$. Stomata's behaviour in opening and closing was observed in both the test and control groups at specified Zeitgeber Times (ZT 0 h, ZT 6 h, ZT 12 h and ZT 18 h).

Distribution of Idioblasts and Crystals from Control and Test Samples

Anatomical and histological studies were conducted on both control and test groups, each consisting of at least ten plants. The stems, leaves, reduced petioles, and flower necks from each group were selected for analysis. Before sectioning, samples were fixed in a solution containing 70% ethanol and 1 part glacial acetic acid, followed by preservation in 70% ethanol. Transverse sections of the samples were obtained using a Leica Biosystems Cryostat (Leica CM 1850, Leica Biosystems Nussloch GmbH, Germany). Subsequently, all sections were stained with 0.1% toluidine blue O for 10 seconds and mounted using glycerol to prepare semi-permanent slides for microscopic examination. The transverse sections were imaged using an Olympus BX 51 light microscope.

Idioblast and Crystal Metrics in Control and Test Samples

The cryostat cut cross-sections ($8 \mu\text{m}$) were stained with 0.1% toluidine blue O and mounted in glycerol. Cell Sens software of the Olympus BX 51 light microscope was used to calculate the length (IL) and width (IW) of each idioblast in each image. The mean idioblast measure for each cross-section was calculated using the average of 5 replicates. The number of idioblasts (ID) per millimetre of section length was calculated. To calculate the index of idioblast expression ($\text{IE} = ((\text{IA}) / \text{total section area}) \times 100$), the IAs were added up and divided by the total cross-sectional area. Total leaf lamina volume minus the percentage of that volume occupied by idioblasts gives the total volume of idioblasts per leaf area (IVA) for each image. Additionally, the number of crystals was calculated. Leaf tissue segments were cut with a microtome for light microscopic examination. 0.1% toluidine blue stain was used to colour these portions. Small leaves were fixed in 2.7% glutaraldehyde for 3 h at 4°C

and then thoroughly washed. The micrographic investigation of the crystal samples was identified by Scanning Electron Microscopy (SEM) (CarlZeiss), and the crystal composition analyses were evaluated with Energy-Dispersive X-ray Spectroscopy (EDS), with an accelerating voltage of 15.0 kV. The z-stacking of idioblasts was performed in the confocal microscope (Leica). The surface scan of idioblasts was micrographed for analysing the surface topography via the tapping mode available in the Innova SPM Atomic Force Microscope. The experimental design regarding idioblasts and crystal matrices was primarily observational and descriptive, focusing on characterizing the morphology and composition of idioblasts in plant leaf tissue using various microscopy techniques and chemical analyses. Whilst there was systematic data collection and analysis involved, the design does not involve the handling of an independent variable or random assignment of subjects to different treatment groups.

Statistical Analysis

Each sample, consisting of triplicate measurements, was derived from 20 individual data sets. Data were expressed as mean $\pm$ standard deviation (SD), with each mean value representing the aggregate of 20 data sets. Significance amongst groups was determined through one-way ANOVA with multiple comparisons, and the number of asterisks in statistical reporting reflected the significance level achieved where one asterisk (*) denotes $p < 0.05$, two (**) denotes $p < 0.01$, three (***) denotes $p < 0.001$ and four (****) denotes $p < 0.0001$, indicating increasing levels of confidence in the observed results.

Results and Discussion

Before examining the impact of melatonin on morphological traits, we quantified its innate levels in both control and test groups in the absence and presence of light. It was consistently found that the test group exhibited higher levels of endogenous melatonin (Fig. 1d). In both the test and control groups, the intrinsic melatonin concentration was highest at ZT 0 h. The maximum melatonin detected in the control was $15,741.61 \text{ ng g}^{-1} \text{ FW}$, and in the test group supplemented with $50 \mu\text{M}$ of melatonin, the maximum content detected was $24,097.22 \text{ ng g}^{-1} \text{ FW}$. Scotophases (ZT 0 & 6 h) experienced a higher melatonin increase than photophases (ZT 12 & 18 h). This has been demonstrated in many studies that the thermal degradation of melatonin is more rapid than photodegradation (Bellmaine et al. 2020; Sadak and Ramadan 2021; Song et al. 2022). Hence, the present study was conducted at a constant temperature due to the rapid thermolability of melatonin. In environments such as

laboratories or experimental chambers, the absence of light can lead to minimal temperature variations. Several factors, including radiative cooling, thermal mass, and air circulation, contribute to these fluctuations when light is removed. Stomatal density and behaviour are reliable proxies for temperature due to their association with transpiration and stomatal conductance (Hasan et al. 2023). Because the number of stomata in plants functioned as a proxy for temperature (Marek et al. 2022; Brewer et al. 2022; Steinhorsdottir et al. 2022), it was critical to investigate how melatonin influenced stomatal behaviour on both the adaxial and abaxial leaf surfaces. The stomata in *P. oleracea* were paracytic (Fig. 1e–m) because the subsidiary and guard cells were situated parallelly. The count was higher on the abaxial surface, and the stomatal appearance was amphistomatic. The density of open stomata hiked at the scotophase in the melatonin-supplemented group (Fig. 2A), but the density of closed and partially closed stomata increased at the photophase (ZT 12 h & 18 h) (Fig. 2B). Melatonin, when administered exogenously, has been shown to improve plant development by increasing net photosynthetic rate (Hu et al. 2022), stomatal conductance, intercellular CO₂ concentration, and transpiration rate (Roy et al. 2021; Wang et al. 2021; Yan et al. 2021; Muhammad et al. 2022), which aligns with the present study. Melatonin-treated plants exhibited increased stomatal density, enhanced scotophase stomatal opening, and photophase stomatal closure. Even though 10 and 50 µM concentrations are occasionally preferred, the 100 µM concentration was determined to be optimum for stomatal density increment, opening and closure. When compared to the control group, high melatonin concentrations at scotophases increased stomata density and facilitated stomatal apertures opening in test groups. In plants, drought is one of the abiotic stressors that hinders growth/development and limits global crop productivity (Phour and Sindhu 2022; Wahab et al. 2022). Stomatal closure is a typical plant adaptation response to water deficit. Stomata govern transpiration/CO₂ uptake by plants. Stomatal guard cells may detect numerous abiotic and biotic stress stimuli from the internal and external environment and respond fast to start closure under unfavourable conditions. Stomata also inhibit the invasion of disease-causing microbes into leaves, limiting their spread. Drought causes the synthesis and/or mobilisation of the phytohormone abscisic acid (ABA), which is well-known for its capacity to induce stomatal closure (Gowtham et al. 2022; Mukherjee et al. 2022). Aside from ABA, plant hormones (auxins, methyl jasmonate, ethylene, brassinosteroids, and cytokinins), plant defensins (salicylic acid and chitosan), and polyamines accumulate during drought and impact stomatal activity (Khare et al. 2020). The role of numerous signalling components/secondary messengers during stomatal opening and closure has been researched extensively. Melatonin appears to act as a supplementary regulator that controls

the process as proven in many studies (Li et al. 2015; Dai et al. 2020; Ahmad et al. 2021; Jiang et al. 2021; Khan et al. 2022). Stomatal movements in the present study were synchronous with foliar nastic movements. Melatonin supplementation increased the number of epidermal cells whilst also increasing the stomatal index (Fig. 3). Thus melatonin was found to act as a mitogen, boosting stomatal ontogeny analogous to auxin (Dai et al. 2020). In plants, higher stomatal density correlates with higher photosynthetic rates (Pathare et al. 2020). *P. oleracea* is a succulent with built-in systems to minimise water loss. As a result, high stomatal density on the leaf's abaxial surface is a natural occurrence. The intriguing aspect was that melatonin appeared to improve the function.

Idioblasts are isolated cells that differ from neighbouring cells in their form, size, contents, and structure, and they can exist in any of a plant's tissue systems. The idioblasts found in *P. oleracea* were highly heterogeneous in nature, occasionally spherical, ovoid or elliptical. Excretory idioblasts are characterised by the accumulation of particular chemicals such as oil, alkaloids, resin, tannin, pigments, or mineral crystals such as calcium oxalate, calcium carbonate, aluminium dioxide and silicon dioxide (Crang et al. 2018). Mineral crystals were always seen to be associated with idioblasts in *P. oleracea*. In most plant families, calcium oxalate raphide/calcium carbonate crystals are found in stellate forms in intravacuolar membrane chambers of specialist idioblasts cells (Pueschel 2019), as discovered in *P. oleracea* of the current study. The degenerating crystal idioblasts of the tobacco anther were found to have two distinguishing traits from normal programmed cell death (PCD) (Liu et al. 2022), notably dynamic alterations in calcium oxalate (CaOx) crystals and the emergence of many peroxisomes. Before PCD, CaOx buildup and H₂O₂ generation happened concurrently or sequentially (Foyer and Hanke 2022). Again idioblasts and crystal content are histological variables that denote a defence mechanism in plants primarily against herbivory (Alencar et al. 2022; Fares et al. 2022). They reflect cellular tonicity patterns governing plants' water relations and thus directly correlate with osmotic potential and turgor pressure (Dubey et al. 2022). Idioblasts were approximately four times more frequent in the leaves of *P. oleracea* than in the stem (Fig. 4B). They were found closer to the leaf vascular tissues, specifically the xylem, and their density varied across the control and test groups (Fig. 4A). The maximum idioblast density was found in the leaf tissues at 50 µM concentration. The density dropped at ZT 12 h when the innate melatonin concentration was reduced. Stem idioblasts grew during the scotophase and decreased during the photophase (Fig. 4B).

The idioblasts were identified using the toluidine blue O stain, indicating the tannins and phenolics' presence. Variation in idioblast size and shape was detected using atomic

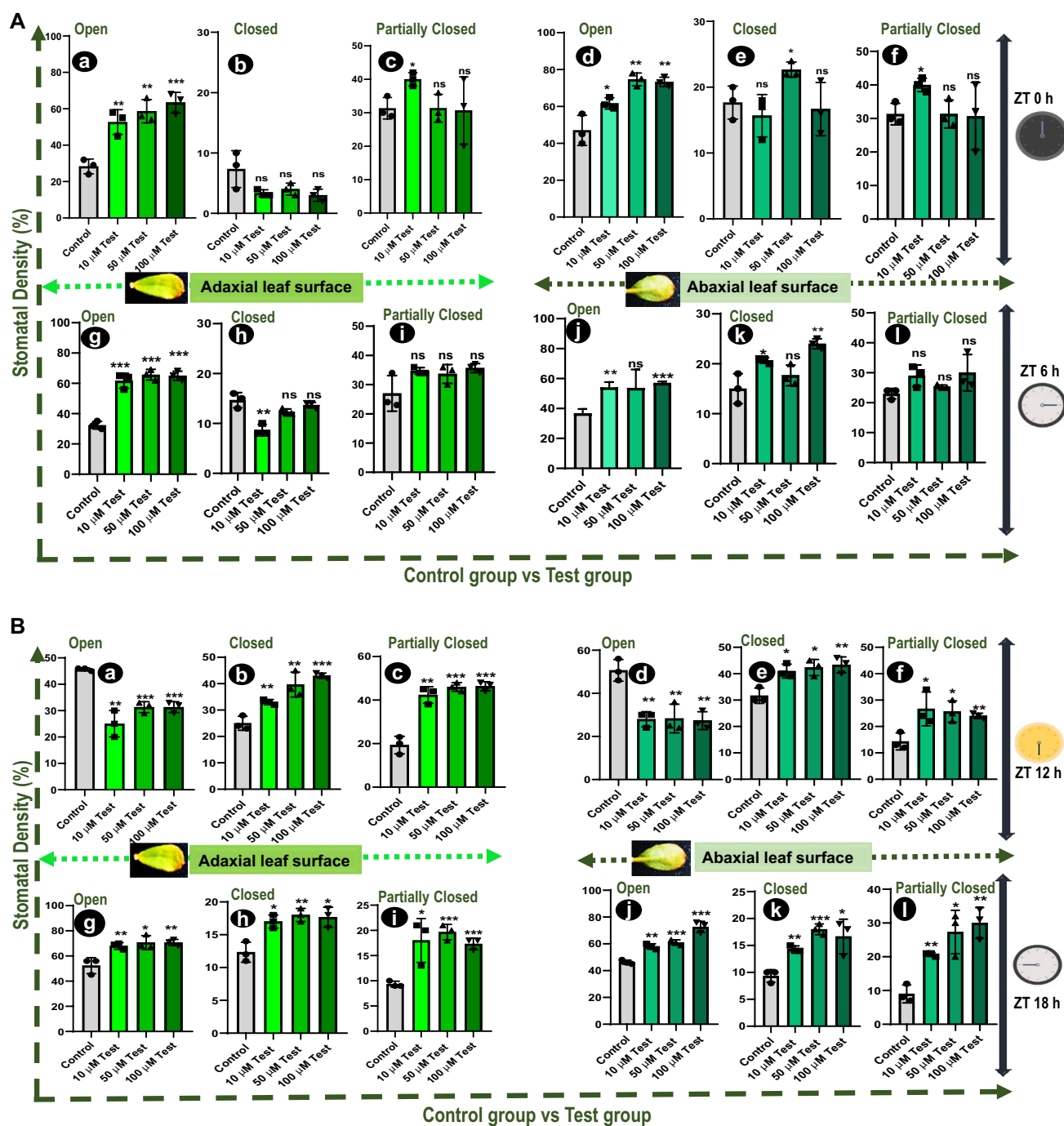


Fig. 2 A Stomatal density (%) Control group vs Test group at specific Zeitgeber Time points. **a, b, c** represent stomatal pore condition at the adaxial leaf surface at ZT 0 h; **d, e, f** represent stomatal pore condition at the abaxial leaf surface at ZT 0 h; **g, h, i** represent stomatal pore condition at the adaxial leaf surface at ZT 6 h; **j, k, l** represent stomatal pore condition at the abaxial leaf surface at ZT 6 h. The three exogenously melatonin concentrations include 10, 50 and 100 μM . The X-axis represents the Control vs Test group (along with melatonin concentration supplemented exogenously). **B** Stomatal

density (%) Control group vs Test group at specific Zeitgeber Time points. **a, b, c** represent stomatal pore condition at the adaxial leaf surface at ZT 12 h; **d, e, f** represent stomatal pore condition at the abaxial leaf surface at ZT 12 h; **g, h, i** represent stomatal pore condition at the adaxial leaf surface at ZT 18 h; **j, k, l** represent stomatal pore condition at the abaxial leaf surface at ZT 18 h. The three exogenously melatonin concentrations include 10, 50 and 100 μM . The X-axis represents the Control vs Test group (along with melatonin concentration supplemented exogenously)

force micrographs (Fig. 4m, n) and confocal microscopy z-stacking (Fig. 4o). The stiffness and number of idioblasts were affected by melatonin administration (Fig. 4A). Melatonin hiked the density of idioblasts in both the stem and the leaves when given exogenously. In contrast, crystals were discovered to be more prevalent in the stem than in the leaf (Fig. 5B). Elemental examination of crystals found in the leaf and stem of *P. oleracea* revealed that they were mostly calcium carbonate and oxalates, with some silicon and aluminium dioxides. The scotophase had the maximum crystal density, whilst the photophase had the lowest. Just like idioblast density, crystal density also hiked at the scotophase. A large number of idioblast cells filled with water in a leaf, in particular, could have several effects on leaf-water relation parameters. Idioblasts potentially alter (1) bulk leaf osmotic potential, (2) the water potential at the turgor pressure deficit loci, (3) hydraulic capacitance, (4) transpiration forces in action, and (5) the hydraulic drifts promoting water uptake in plants (Sruthi and Puthur 2020; Arsic et al. 2022). Transpiration pull, water capacitance and osmotic potential are remarkably crucial for succulents.

Idioblasts and crystals have rarely been demonstrated as contributing to foliar water uptake in angiosperms (Bazzicalupo et al. 2021; Vitarelli et al. 2021; Mehlreter et al.

2022). The succulent idioblasts encompass mucilaginous contents, which cause hydraulic redistribution in tissues in the presence of temperature and light. The hydraulic forces are primarily responsible for rendering shapes to idioblast cells (Pfadenhauer and Klötzli 2020). Crystals being tiny, get navigated across the leaf locales through the hydraulic shifts across vascular tissues. Higher idioblast and crystal contents bring hypertonicity, promoting water influx (hyperhydricity). Moving idioblasts facilitate water redeployment in cells. The presence of water influx in cells leads to the opening of the leaves during photophase. To prevent excessive water loss through transpiration during photophase, the stomatal guard cells become flaccid, transferring water to the subsidiary, contouring epidermal cells, and shutting the stomatal pore. During the scotophase, idioblasts and crystal count in the leaf strictures decrease, resulting in water outflow. One of the most effective ways the plant accomplishes this is by encouraging transpiration via the leaves. During the scotophase, water loss through open stomatal holes (turgid guard cells) causes weight loss from the leaf lamina, resulting in turgor pressure reduction. A decrease in turgor pressure at the shortened petiolar zone facilitates inward leaf closure. The abaxial side has the highest stomatal density. Melatonin supplementation augmented these hydrodynamic functions

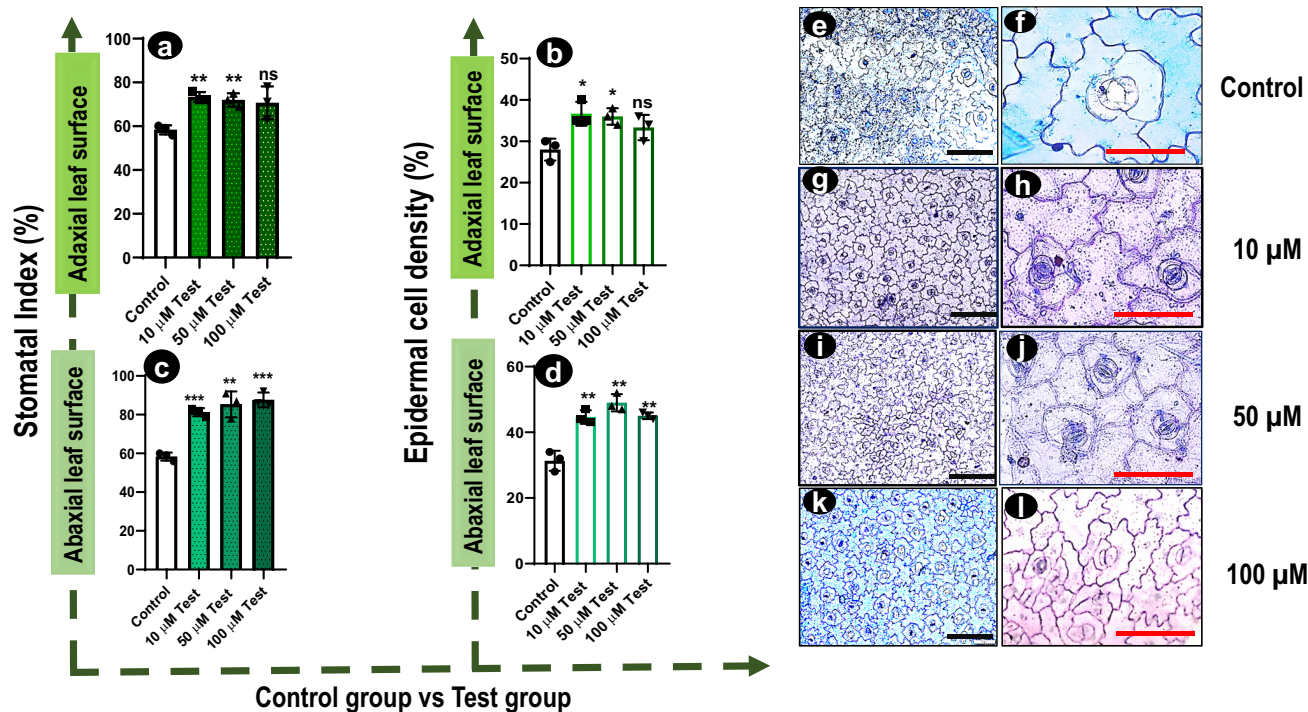


Fig. 3 Stomatal index (%) and epidermal index (%) of the Control group vs the Test group. **a** represents stomatal index **b** represents epidermal index as calculated in the adaxial leaf surface **c** represents stomatal index **d** represents epidermal index as calculated in the abaxial leaf surface; **e** and **f** represent stomatal features of the Control group;

g and **h** represent stomatal features of the Test group supplied with 10 μ M melatonin; **i** and **j** represent stomatal features of the Test group supplied with 50 μ M melatonin; **k** and **l** represent stomatal features of the Test group supplied with 100 μ M melatonin. Scale bar = 100 μ m

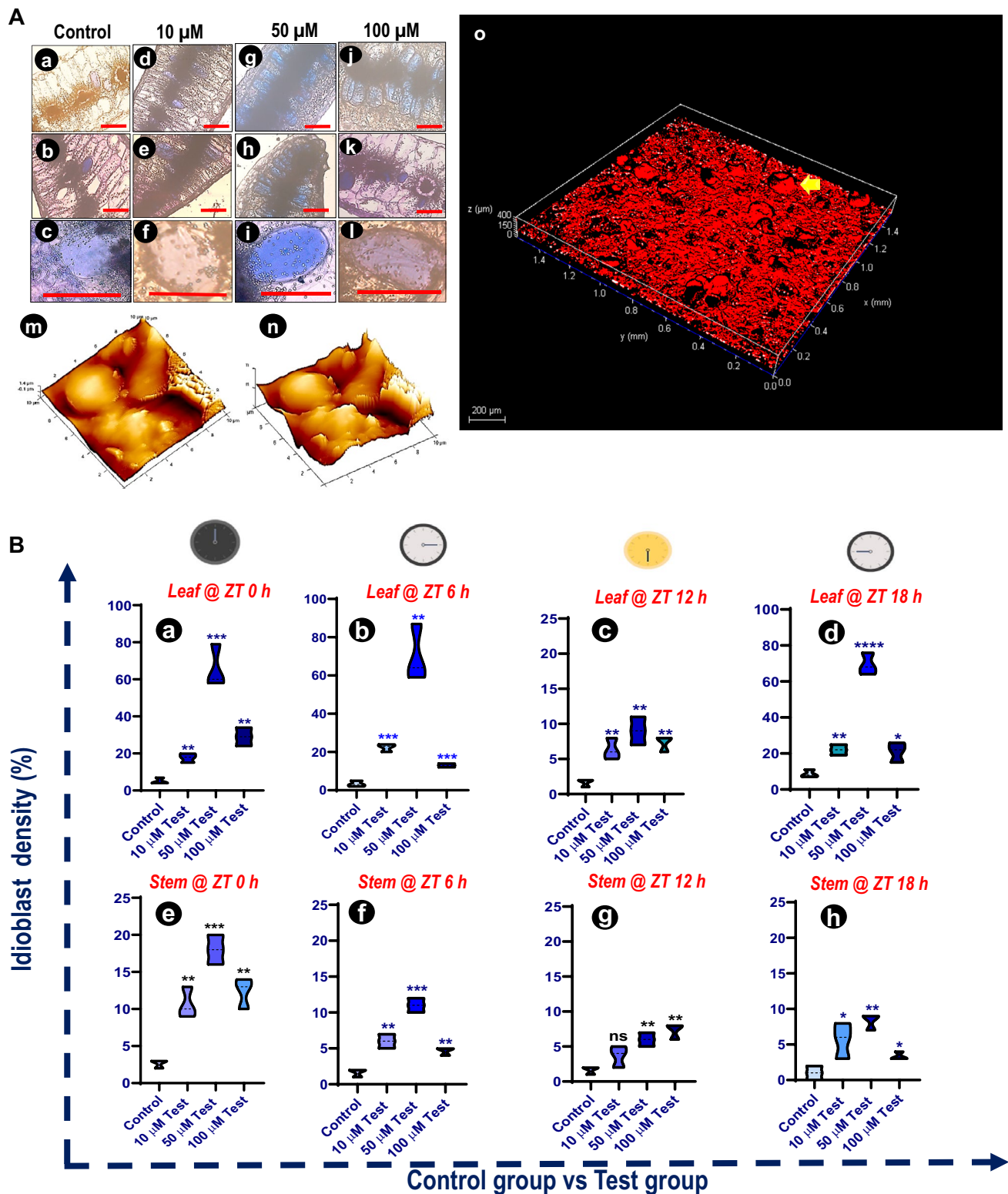


Fig. 4 **A** Idioblast features present in *P. oleracea* Control group vs Test group. **a, b** and **c** represent idioblasts present in the Control group; **d, e, f** represent idioblasts present in the Test group supplemented with 10 μM of melatonin. **g, h, i** represent idioblasts present in the Test group supplemented with 50 μM of melatonin. **j, k, l** represent idioblasts present in the Test group supplemented with 100 μM of melatonin. Scale bar = 500 μm . **m, n** represent atomic force micro-

graphs of idioblasts; **o** represent z-stacking performed using confocal microscopy for leaf surface to capture idioblasts. **B** Idioblast density (%) in *P. oleracea* Control group vs Test group in leaf and stem at specific Zeitgeber Time points. Leaf idioblast density in the Control vs Test is represented at **a** ZT 0 h **b** ZT 6 h **c** ZT 12 h **d** ZT 18 h. Stem idioblast density in the Control vs Test is represented at **a** ZT 0 h **b** ZT 6 h **c** ZT 12 h **d** ZT 18 h

by navigating both idioblasts and the stellate crystals across tissues causing increment in osmotic potential as indicated in studies elsewhere (Eisa et al. 2023; Jalili et al. 2023; Kebbeh et al. 2023; Luo et al. 2023; Sheikhalipour et al. 2023). The petiole of *P. oleracea* is highly reduced but contains globular cells which function analogous to that of a conveyor zone trafficking idioblasts and crystals via vascular elements. It is seen that idioblast abundance was always near the vascular tissues, specifically around xylem vessels (Sack and Scofoni 2013). The idioblasts' ovoid shape and heterogeneous dimension suggest cellular hydraulic shifts steer them across tissues. The mucilaginous content from the secretory cells alleviates the frictional forces that tend to occur during the idioblast–crystal transits.

In summary, melatonin promotes hyperhydricity in the tissue milieu via traversing idioblasts and crystals. Melatonin levels rise during the scotophase, increasing hydricity and turgor pressure and resulting in foliar inward closure. Stomatal opening promotes transpiration pull, which increases hydrodynamicity in the leaf lamina. Melatonin levels fall during photophase, reducing hydricity and idioblast/crystal density and decreasing hypertonicity in the leaf lamina. To preserve water, leaf stomata close at this point, slowing transpiration. Water redeployment in the tissue promotes the opening of the leaf lamina, allowing for photosynthesis. At scotophase, injection of 50 μM melatonin enhanced the density of both idioblasts and crystals. Thus, crystal and idioblast serve as visual indicators for the underlying cause of hyperhydricity and turgor pressure. The entry and exit of idioblasts/crystals are explicitly channelled by melatonin, influencing turgor pressure and thereby favouring nastic motions. This indisputably demonstrates that melatonin plays a direct and pivotal role in enhancing tissue hydricity and elasticity. 100 μM melatonin was effective in increasing stomatal density. The concentration of 100 μM was most likely ideal for eliciting mitogenic activity in *P. oleracea* stomatal cells.

In the physical sciences, it is well-established that the kinetics of a rigid entity involve a combination of translational and rotational motion. However, when fixed along a line, such as the petiolar connection of a leaf lamina, the motion becomes solely rotational. This analogy can be equated to a door hinge, where the leaf lamina acts as the door. The force required to induce rotational motion is applied perpendicular to the lamina, typically from the lamina's tip towards the midrib zone. This force, referred to as petiolar torque, is generated by the movement of idioblast–crystal entities, as depicted in the graphical abstract. This torque is exerted towards the petiolar hinge due to hydraulic pull resulting from transpiration during the scotophase, leading to inward leaf closure. Concurrently, stomatal pores open to facilitate transpiration. Conversely, when the direction of force reverses (referred to as lamina torque),

hydraulic push redistributes water within the leaf lamina, resulting in leaf opening. This cyclic process of torque generation and response to hydraulic forces governs the dynamic opening and closing movements of leaves. At this instant, the stomata pore closes to conserve water, facilitating photosynthesis. Swollen cells at the leaf tip and petiolar zone of *P. oleracea* contribute to water retention/depletion, consequently leading to turgor pressure gain/loss triggering hydraulic shifts. The inward closing of *P. oleracea* leaves a typical example of rotational motion in a fixed axis. Because the axis is fixed, only torque components acting in the direction of the fixed axis must be addressed. The hydraulic tension exerted on idioblasts and crystals causes the leaves to follow a circular path inward, with a petiolar base at the centre of the axis. This rotating motion is additionally accelerated by water loss due to transpiration pull during the scotophase. Because of mucilaginous secretions within the leaf lamina, friction during rotational motion is significantly decreased. As a result of the lack of friction amongst particles, the possibility of external torque about the axis of rotation is skewed. Melatonin thus plausibly regulates plant gnathophysiology by controlling the hydricity in tissues.

Plant anatomy is a meticulous exploration of the internal morphology of plants, unravelling the intricate spatial arrangement and composition of tissues, cells, and organs. This investigation spans the macroscopic features visible to the naked eye, as well as the microscopic details discernible only under magnification, including the delicate structures of stomata. Within the realm of plant anatomy lies plant histology, a specialized domain focused on the microscopic examination of plant tissues. Here, the cellular architecture, arrangement, and physiological roles of tissues are meticulously delineated. Through histological inquiry, thin sections of plant tissues are scrutinized under magnification, allowing for the precise identification and characterization of cell types, cell wall compositions, and specialized structures. In this context, our current study specifically targets the localization and spatial distribution patterns of idioblasts and crystals within plant tissues. Together, plant anatomy provides a sweeping panorama of plant structures, whilst plant histology delves deep into the nuanced intricacies of tissue morphology. This amalgamation of disciplines enriches our understanding of plant form and function, showcasing their synergistic interplay in enigmatic botanical mechanisms, such as the cryptic nature of nastic movements.

Conclusion

Melatonin emerges as a fundamental signalling molecule influencing turgor dynamics within specific plant tissues, implicating idioblast and crystal densities as putative determinants in orchestrating the idiosyncratic foliar nastic

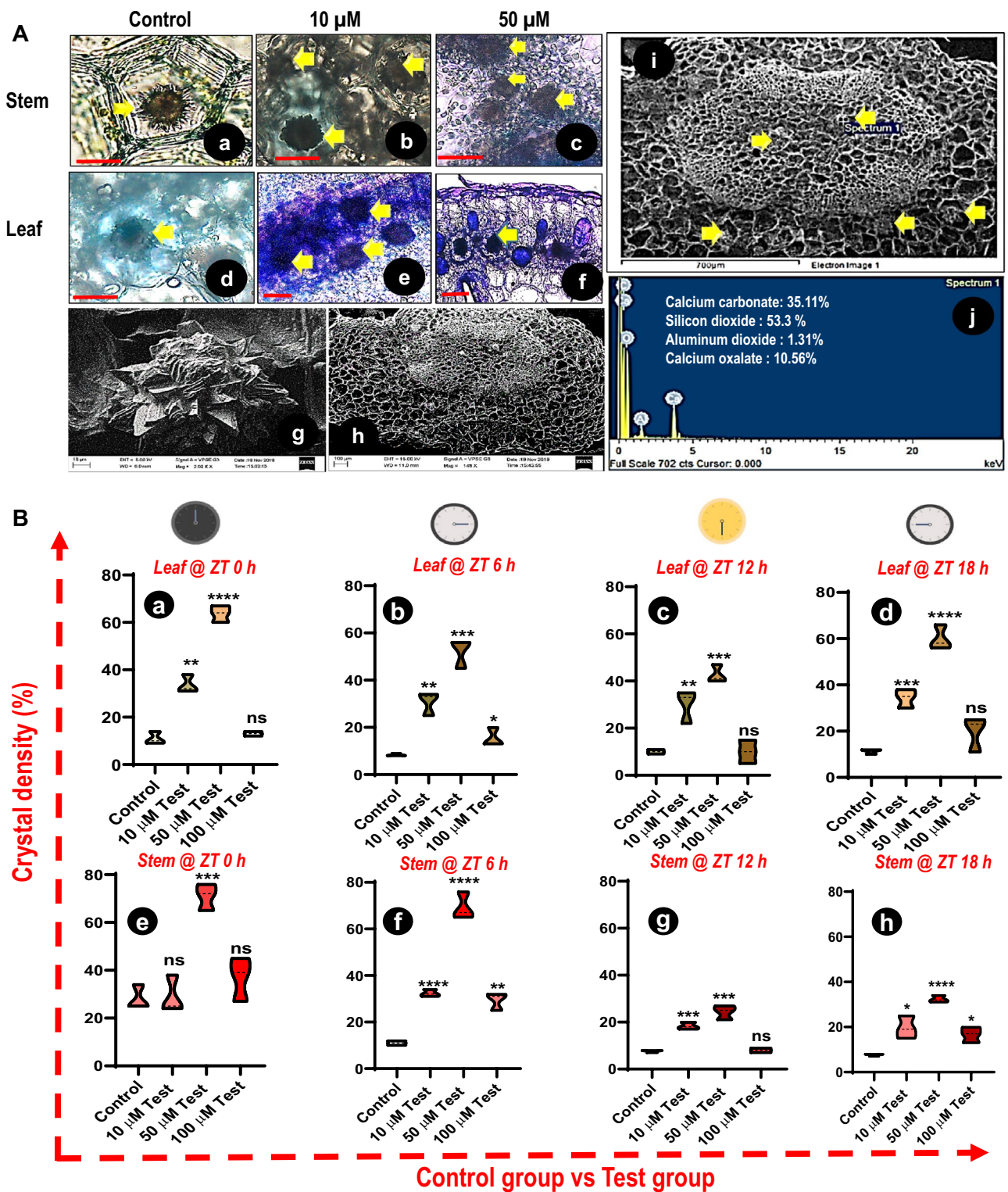


Fig. 5 **A** Crystal features present in *P. oleracea* Control group vs Test group. **a** represents crystal present in the stem Control group; **b** and **c** represent crystal present in the stem Test group supplemented with 10 μM and 50 μM melatonin, respectively. **d** represents crystal present in the leaf Control group, **e** and **f** represent crystal present in the leaf Test group supplemented with 10 μM and 50 μM melatonin, respectively. Scale bar = 500 μm . **g** and **h** represent scanning electron micrograph of crystal found in the stem. **i** represent the stem section

scanned for EDX analyses for crystal elemental analyses via scanning electron microscopy. **j** represent elements detected in *P. oleracea* via EDX analysis. **B** Crystal density (%) in *P. oleracea* Control group vs Test group in leaf and stem at specific Zeitgeber Time points. Leaf crystal density in the Control vs Test is represented at **a** ZT 0 h **b** ZT 6 h **c** ZT 12 h **d** ZT 18 h. Stem crystal density in the Control vs Test is represented at **e** ZT 0 h **f** ZT 6 h **g** ZT 12 h **h** ZT 18 h

movement of *P. oleracea*. Nastic movements, representative of non-directional plant responses to external stimuli, are usually elicited by an array of environmental triggers such as light, temperature, and tactile stimuli. In *P. oleracea*, leaf movement manifests predominantly through the agency of hydraulic and mechanical forces. Notably, the petiolar connection of the leaf lamina assumes a central role akin to a hinge, where the precise application of force triggers rotational motion along a fixed axis. This rotational dynamics, facilitated by idioblast–crystal entities, orchestrates the nocturnal closure of leaves under the force of hydraulic pull stimulated by transpiration, whilst diurnal leaf expansion results through hydraulic push, facilitating photosynthesis and water conservation through stomatal closure. The leaf movement in *P. oleracea* thus mirrors the essence of nastic responses, characterized by non-directional responses to stimuli. The inward leaf closure, propelled by petiolar torque and hydraulic tension on idioblasts and crystals, epitomizes a unique bio-model of rotational motion, with the petiolar base serving as the swivel. Augmented by transpiration-induced water loss and facilitated by mucilaginous leaf secretions, this rotational motion surpasses frictional hindrances, illustrative of temperature-driven phenomena. Melatonin emerges as a pivotal regulator of plant gnosis-physiology, modulating tissue hydricity amidst its myriad physiological roles, albeit its precise molecular mechanisms warrant further elucidation. The intricacies of leaf movement emphasize the interplay of hydraulic and mechanical forces, accentuated by the confluence of abiotic factors such as light and temperature in controlling melatonin levels within *P. oleracea*. Understanding the gradations of idioblast and crystal torques and their angular momentum promises insights into the pleiotropic effects of melatonin and the mechanisms of foliar nastic movements across diverse succulent taxa.

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Data Availability This published article and its supplementary information files include all data generated or analyzed during this study.

Declarations

Conflict of interest The authors declare no competing/conflicting interests.

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