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Research Paper

Warm Nights, Waxy Leaves: Exploring the Interplay Between Epicuticular Wax and Nocturnal Transpiration

Nagaraju SPOORTHI[1], Nedaravalli Manjappa KAVYASHREE[1], Vedamurthy SUSHMA[1],
Preethi VIJAYARAGHAVAREDDY[1, 2], Sheshshayee SREEMAN[1]
(*[1]Department of Crop Physiology, University of Agricultural Sciences, GKVK, Bengaluru 560065, India; [2]Centre for Sustainability, Chanakya University, Devanahalli 562110, India*)

Abstract: High epicuticular wax (E_{wax}) content on leaves is a key trait for drought and heat stress tolerance in plants. While E_{wax} reduces daytime water loss by reflecting solar radiation and maintaining cooler canopy temperatures, its role in nocturnal transpiration remains less understood. Using phenomics, we identified a balance between cuticular and stomatal transpiration at night, with wax modulating these processes under varying night temperatures. Significant variability in E_{wax} content, nocturnal transpiration, and related morpho-physiological traits across genotypes highlights the complexity of these mechanisms. Seasonal and environmental factors, particularly temperature, strongly influence E_{wax} accumulation, with higher levels during warmer nights. Our data reveal a positive correlation between E_{wax} content and nocturnal mean transpiration rate, suggesting that genotypes with higher wax levels may open stomata to dissipate heat while minimizing water loss. Notably, genotypes with high wax content showed a lower increase in transpiration during warmer nights compared with cooler ones, indicating a regulatory role of wax under elevated temperatures. This research provides insights into the dynamics of E_{wax} and nocturnal transpiration, offering strategies to enhance plant resilience to rising night temperatures. Such findings are critical for breeding climate-resilient crops, advancing sustainable agriculture, and ensuring food security.

Key words: epicuticular wax; high temperature; nocturnal transpiration; phenomics; rice

The Earth's warming trend has intensified significantly since 1982, accelerating to about 0.20 ºC per decade. Consequently, 2023 has been confirmed as the warmest year on record, with global temperatures approximately 1.18 ºC above the 20th-century average (Berkeley Earth, 2023; NASA, 2023; NOAA, 2023). Such rising global temperatures pose substantial challenges, profoundly impacting agricultural systems, and highlighting the urgent need for research into the effects of heat stress on plants and the adaptations they employ to survive in increasingly hostile environments (NASA, 2021).

Plants exhibit adaptive growth responses to environmental stimuli, with temperature emerging as a pivotal factor. Each plant species flourishes within a delineated temperature range that optimally supports its development (Ohtaka et al, 2020). However, high-temperature stress detrimentally impacts various cellular, anatomical, and physiological processes in crop plants, ultimately resulting in a yield reduction (Wahid et al, 2007; Nievola et al, 2017; Sadok et al, 2021; Yang et al, 2023). Zhao et al, 2017 reported that with each 1 ºC increase in global temperature, the yields of major crops such as maize, wheat, rice, and soybean are expected to drop by 7.4%, 6.4%, 3.2%, and 3.1%, respectively. To mitigate this thermal stress, plants endeavour to maintain a cooler canopy through an important physiological process known as

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Corresponding authors: Preethi VIJAYARAGHAVAREDDY (preethi.v@chanakayauniversity.edu.in); Sheshshayee SREEMAN (msshesh1@uasbangalore.edu.in)

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transpiration (Desai et al, 2021). Weerakoon et al (2008) discovered that rice plants can maintain the microclimate of their canopy below the critical threshold of 33 ºC through effective transpiration cooling, even when external temperatures exceed 40 ºC, if there is sufficient water and low humidity levels. It is well-established that plants transpire during both diurnal and nocturnal periods, with the rate of transpiration primarily dictated by temperature and vapor pressure deficit (VPD) during these times (Caird et al, 2007; Dawson et al, 2007; Sreeman et al, 2018). In addition to external factors, intrinsic characteristics such as wax content, cuticle thickness, and other anatomical traits significantly influence the regulation of transpiration (Riederer and Schreiber, 2001). The role of waxes in regulating the canopy temperature and transpiration has been well documented in several species (Blum, 1975; Holmes and Keiller, 2002). A recent study by Carvalho et al (2020) clearly demonstrated the vital role of cuticular wax content in regulating plant canopy temperature under both abundant and limited water conditions, highlighting the importance of waxes in plant thermal regulation.

Of total transpiration, nocturnal transpiration constitutes about 5%–15% in most cases, however it can surpass 50% depending on the species and environmental conditions (De Dios et al, 2015; Fricke, 2020; Yi and Yano, 2023). The phenomenon of nocturnal transpiration has been well explored and its significance in regulation of water and nutrient accessibility has been comprehensively elucidated across diverse plant species (Caird et al, 2007; Fricke, 2019; Spoorthi et al, 2024). Rice, a crop with high water demands (Jarin et al, 2024), requires a deeper understanding of its water dynamics. Researchers are particularly interested in quantifying nocturnal transpiration and its effects on physiological processes, as this knowledge could enhance water management strategies and boost rice crop resilience

Although numerous studies (Bahuguna et al, 2022; Yi and Yano, 2023; Yan et al, 2023) have been conducted to understand and quantify nocturnal transpiration in rice, most have focused on measuring total nocturnal transpiration by aggregating water loss over the entire night. While a limited number of studies have examined nocturnal transpiration on an hourly basis but under controlled environmental conditions. However, there exists significant differences between controlled and natural environments; and hence the results are not comparable (Impa et al, 2021). This gap highlights a lack of understanding of rice night transpiration trend in natural conditions. Additionally, accurately measuring the precise volume of water lost at the plant canopy level on an hourly basis in natural conditions demands sophisticated phenomics platforms. In our research, we harnessed a top-tier phenomics facility equipped with an automated irrigation system that tracks nocturnal transpiration hourly, as described by Vijayaraghavareddy et al (2020a). This innovative approach enabled us to meticulously document variations in nocturnal transpiration in response to fluctuations in night temperatures. In this study, we hypothesize that while epicuticular waxes play a significant role in mitigating heat load and acting as a barrier to water loss, their impact under non-radiative conditions, such as nighttime when stomata are closed, may be counterproductive. Specifically, reduced cuticular transpiration resulting from high wax deposition could lead to an accumulation of heat within the leaf tissue. This increased heat load may potentially trigger the reopening of stomata, thereby enhancing nocturnal transpiration.

RESULTS

Variability in morpho-physiological traits among the contrasts for epicuticular wax

Six contrasting genotypes with varying epicuticular wax (E_{wax}) content, ranging from 5.2 to 16.7 μg/cm^2, were selected from an initial experiment (Table S1). These genotypes were subsequently regrown in the phenomics facility, aiming to understand nocturnal transpiration. Total leaf area (TLA) among the genotypes exhibited significant variation, with the highest TLA observed in IRG 277 and the lowest in IRG 066 (Fig. 1-A). Similarly, total dry matter (TDM) was greatest in both IRG 277 and IRG 066 (Fig. 1-B). Genotypes selected for high E_{wax} content demonstrated increased levels, while those selected for lower wax content showed minimal accumulation in consistent with the previous experiment. Interestingly, the medium wax genotype, IRG 355, exhibited E_{wax} levels comparable to high wax genotypes (Fig. 1-C). Scanning electron microscopy also revealed consistent wax accumulation, corroborating the quantitative data (Fig. S1). When assessing the seasonal impact on TDM and E_{wax}, the hotter summer of 2024 than monsoon 2023, led to a decline in TDM and a concomitant increase in E_{wax} content. The reduction in TDM was most pronounced in the low-wax genotype, IRG 335 and minimal in IRG 263, a medium-wax genotype.

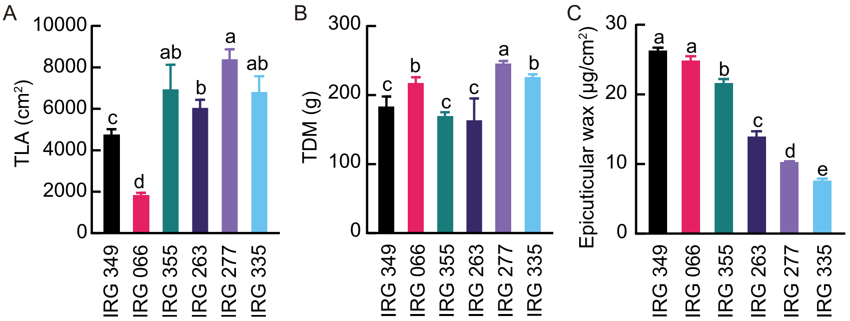

Fig. 1. Variability in morpho-physiological traits among rice genotypes with differing epicuticular wax (E_{wax}) content.

TLA, Total leaf area; TDM, Total dry matter measured at harvest.

Different lowercase letters above the bars indicate significant differences at $P < 0.05$ by the one-way ANOVA was conducted. The rice genotypes IRG 349 and IRG 066 are high E_{wax} types; IRG 355 and IRG 263 are moderate E_{wax} types, while IRG 277 and IRG 335 are genotypes with low E_{wax} content.

Additionally, E_{wax} content nearly doubled in 2024 relative to 2023 because of higher temperature, with the greatest increase recorded in IRG 335 and the smallest in IRG 263 (Tables S2 and S3).

Variability in nocturnal transpiration

The genotypes exhibited substantial variability in nocturnal transpiration, ranging from a high of 15.2 L/pot to a low of 8.1 L/pot (Fig. 2-A). Notably, only genotypes with high E_{wax} content displayed distinct differences in nocturnal transpiration, while other genotypes showed no significant variation. All genotypes transpired more during the early night period compared to the late-night period (Fig. 2-B and -C). Similarly predawn transpiration also showed consistent pattern. Upon normalizing leaf area to determine the mean transpiration rate (MTR), the low-transpiring genotype IRG 066, because of less total leaf area, exhibited a high MTR, similar to the high-transpiring genotype IRG 349. In contrast, no significant differences in MTR were observed among the other genotypes (Figs. 2-E to -H).

Correlation analysis between various parameters

E_{wax} exhibited a positive correlation with MTR but not with CWT. Among the MTR measurements, both MTR during the late night and predawn showed a strong

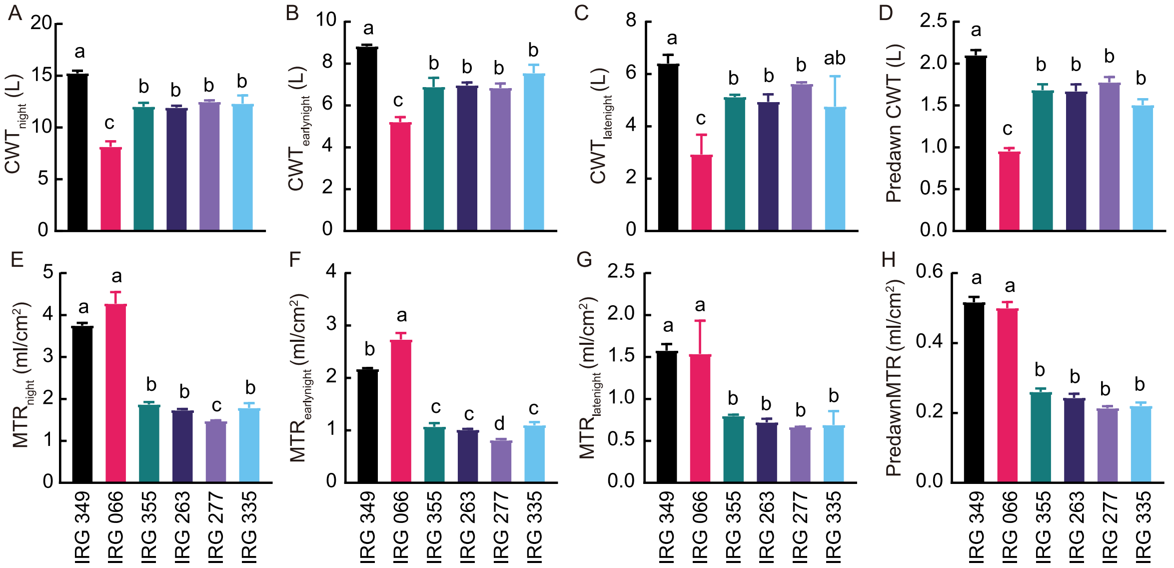

Fig. 2. Variability in transpiration parameters among rice genotypes with differing epicuticular wax (E_{wax}) content.

A, Cumulative water transpired (CWT) over the entire night (18:00 to 06:00, CWT_{night}); **B**, CWT during the early night period (18:00 to 00:00, $CWT_{earlynight}$); **C**, CWT during the late-night period (00:00 to 06:00, $CWT_{latenight}$); **D**, CWT during the predawn hours (04:00 to 06:00, Predawn CWT), measured from 20 to 100 d after sowing. CWT per unit leaf area (MTR) was calculated for the entire night (**E**, MTR_{night}), early night (**F**, $MTR_{earlynight}$), late night (**G**, $MTR_{latenight}$), and predawn hours (**H**, Predawn MTR).

Different lowercase letters above the bars indicate significant differences at $P < 0.05$ by the one-way ANOVA was conducted. IRG 349 and IRG 066 are genotypes with higher E_{wax} types, IRG 355 and IRG 263 are ones with moderate E_{wax} types, while IRG 277 and IRG 335 are ones with lower E_{wax} content.

correlation with each other. However, TDM did not correlate with any of the variables, while TLA showed a strong negative correlation with E_{wax}. Further, TLA had no correlation with CWT night, across various nighttime hours (Fig. S2). Similarly, TDM showed no association with CWT or MTR, whether during the night, late night, or early night hours. CWT during the early night was strongly correlated with CWT in the late night (Fig. S2).

Effect of increasing temperature on night transpiration

We observed varying minimum temperatures during the night hours and pooled the transpiration data from those nights to assess its impact (Figs. S3 and S4). While slight temperature increases (from 20.0 ºC to 20.2 ºC and 20.4 ºC) led to minor changes in nighttime transpiration for some genotypes, the most notable differences occurred when temperatures exceeded 21.0 ºC (Fig. 3). This effect was more pronounced in low-wax genotypes compared with high-wax genotypes, as indicated by their steeper slope values (Table S3). When analysing hourly transpiration across different temperatures, the early night and predawn periods displayed substantial variation compared transpiration at middle of night. At the highest recorded minimum night temperature (21.8 ºC), compared with the lowest (20.0 ºC), the increase in CWT during the early night period was greater in all genotypes except IRG 066, which showed an increase during the late night and predawn hours (Fig. 4). Among the genotypes, low E_{wax} types exhibited a higher increase in transpiration compared with medium and high E_{wax} types. Within the low E_{wax} group, IRG 277 had a more significant increase during the early night hours, followed by a reduced increase after midnight, whereas IRG 335 showed a steady increase throughout the entire night (Fig. 4).

Effect of epicuticular wax and nighttime temperature on nocturnal transpiration

A regression analysis was conducted to examine the percent increase in CWT$_{\text{night}}$ at each temperature increment compared with 20.0 ºC (Fig. 5). At the initial temperature increases (20.0 ºC, 20.4 ºC, 20.6 ºC, 20.8 ºC, and 21.0 ºC), E_{wax} showed a negative but non-significant association with the percent increase in CWT$_{\text{night}}$. However, beyond 21.0 ºC, E_{wax} demonstrated a strong and significant negative association. This association was particularly pronounced at temperatures of 21.4 ºC and 21.6 ºC (Fig. 5).

DISCUSSION

The novel Phenomics facility at UASB has the unique advantage of determining transpiration in real-time. We have demonstrated that plants lose a significant volume of water during the night period with a pre-dawn burst (Spoorthi et al, 2024). Recently, there has been a significant increase in understanding the importance as well as the regulation of nocturnal transpiration, which have largely been theoretical. It is well known that epicuticular wax ($E_{\text{wax)}}$ load keeps the canopy cool and determine transpiration rates during day times. Little was known about the role of waxes on leaf surface in determining nocturnal transpiration.

Our results provide valuable insights into the role of E_{wax} in modulating nocturnal transpiration, particularly under varying night temperature conditions. The

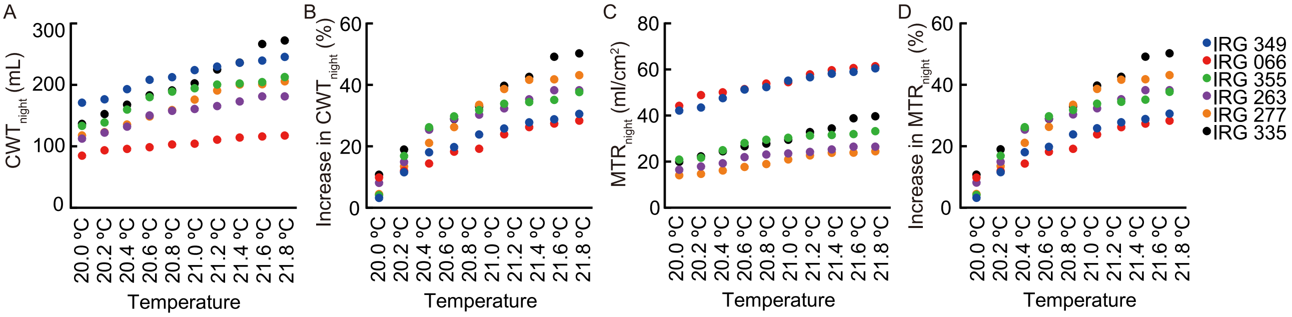

Fig. 3. Variability in transpiration among rice genotypes with differing epicuticular wax (E_{wax}) content at varying night temperatures.

A, Cumulative water transpired (CWT) over the entire night (18:00 to 06:00, CWT$_{\text{night}}$); **B**, Percentage of increase in CWT at different temperatures compared with the lowest temperature of 20 ºC; **C**, CWT per unit leaf area (MTR) was calculated for the entire night; **D**, Percentage of increase in MTR at different temperatures compared with the lowest temperature of 20 ºC.

Data were collected on days corresponding to these respective temperatures. IRG 349 and IRG 066 are genotypes with higher E_{wax} types, IRG 355 and IRG 263 are ones with moderate E_{wax} types, while IRG 277 and IRG 335 are ones with lower E_{wax} content.

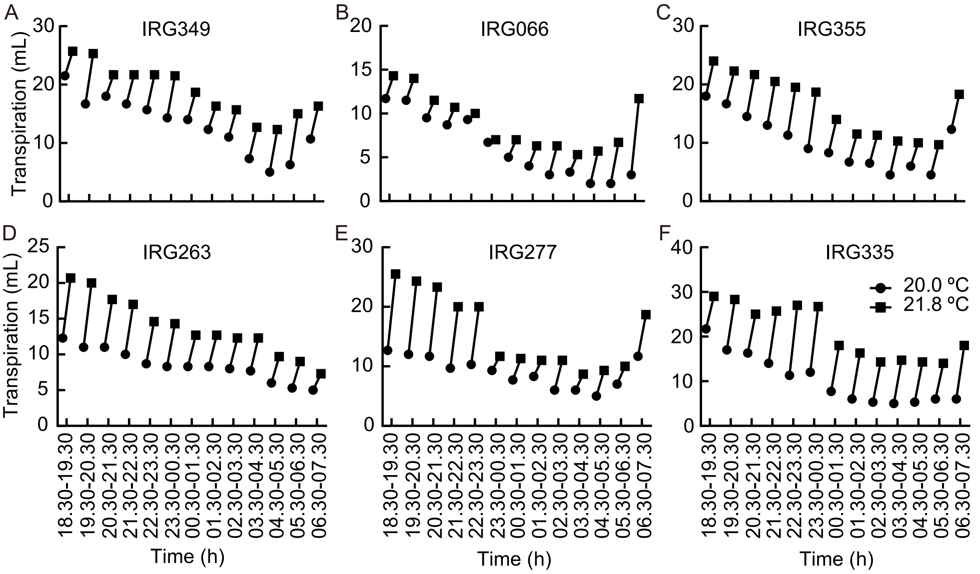

Fig. 4. Variability in transpiration among rice genotypes with differing epicuticular wax (*E*$_{wax}$) content at two different night temperatures.

Data were collected on two consecutive days: April 6th, day with a minimum night temperature of 20 °C, and April 7th, day with a maximum night temperature of 21.8 °C. IRG 349 and IRG 066 are genotypes with higher *E*$_{wax}$ types, IRG 355 and IRG 263 are ones with moderate *E*$_{wax}$ types, while IRG 277 and IRG 335 are ones with lower *E*$_{wax}$ content.

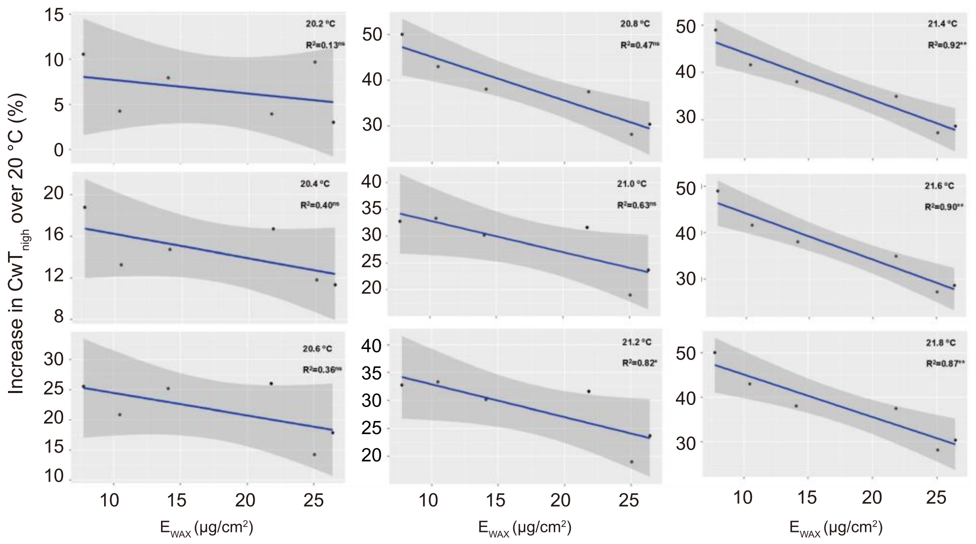

Fig. 5. Regression analysis between epicuticular wax content (*E*$_{wax}$) and percentage reduction in cumulative water transpired during night (CWT$_{night}$) at different night temperatures, compared with 20.0 ºC.

substantial variability observed among genotypes for *E*$_{wax}$ content, nocturnal transpiration, and other morpho-physiological traits highlights the complexity of plant responses to environmental stress, particularly to increasing night temperatures.

Cuticular transpiration and stomatal transpiration trade-off

Plants exhibit both stomatal and cuticular transpiration during the night (Caird et al, 2007). While cuticular transpiration generally represents a small portion of total water loss, it can become more prominent when stomata are closed, as typically observed during nighttime or when plants are subjected to stress. Under conditions of high temperatures and low humidity or moisture stress, cuticular transpiration can increase due to the steep gradient between moisture within the leaf and the drier surrounding atmosphere (Shellakkutti et al, 2022). Epicuticular wax accumulation plays a crucial role in reducing the heat load during the day by reflecting solar radiation, thereby creating a cooler canopy (Holmes and Keiller, 2002). This reflective property is particularly important in environments with high radiation, as it helps to prevent excessive heat absorption, leading to lower transpiration rates and improved regulation of leaf temperature during daylight hours. By minimizing daytime water loss, waxy surfaces enhance the plant's ability to conserve water under hot conditions (Lewandowska et al, 2020). However, the situation might be completely different during the night. The reduction in cuticular water loss, due to increased accumulation of epicuticular wax (Shepherd and Griffiths, 2006), can cause an imbalance in the plant's water relations during nights. With restricted cuticular transpiration, internal water pressure may build up, prompting the plant to open its stomata to release water. This mechanism could lead to increased nocturnal transpiration, as the plant seeks to balance its internal moisture levels. Furthermore, in warmer nights, this effect could be exacerbated as the plant attempts to cool itself through increased stomatal water loss. During the early night, the difference in transpiration between low and high night minimum temperatures was significantly greater compared with later in the night, when temperatures were slightly cooler (Fig. 4). In this way, nocturnal transpiration could serve as an alternative cooling mechanism (Coupel-Ledru et al, 2016), compensating for the limited water loss through the cuticle due to the presence of higher levels of epicuticular wax. This dynamic pattern reveals an intricate trade-off between cuticular and stomatal transpiration during the night. Our data, shows a positive correlation between epicuticular wax accumulation and MTR during the night, highlights this delicate balance (Fig. S2). Although it has been demonstrated that enhanced wax can reduce both daytime and nighttime transpiration (Richard et al, 1986), this effect might only hold true under optimal nighttime temperatures. Our experiments, conducted during the high-temperature conditions of summer with naturally fluctuating environments, revealed a positive correlation between wax content and mean nocturnal transpiration rate (Fig. S2). While wax accumulation is advantageous for minimizing water loss during day, it may drive plants to rely on stomatal transpiration at night.

Role of epicuticular wax in reducing water loss at high night temperatures

High epicuticular wax genotypes demonstrate increased stomatal transpiration at night, yet they experience a reduction in overall transpiration when exposed to fluctuating high night temperature conditions. Epicuticular wax serves as a hydrophobic barrier on leaf surfaces (Koch and Ensikat, 2008), significantly limiting cuticular transpiration. This waxy layer prevents water from escaping directly through the epidermal cells, which is especially important at high temperatures and/or dry weathers (Rahman et al, 2021). In our study, although MTR and E_{wax} content showed a positive association, the presence of these waxes contributed to reduced transpiration rates during warmer nights. Specifically, the percentage increase in transpiration for genotypes with high E_{wax} content was significantly lower on nights when the minimum temperature exceeded 21 ºC (Fig. 3). This suggests that under well-watered, normal conditions, stomata tend to remain open during the night, facilitating stomatal transpiration. This effect was more pronounced in high-wax genotypes, likely because their waxy leaf surface aids in canopy cooling through stomatal transpiration.

Elevated nighttime temperatures often prompt greater stomatal opening to facilitate transpiration and provide cooling (Sadok and Jagadish et al, 2020) this was prominent in low wax genotypes. However, as nighttime temperatures rise beyond the optimal range, the stomata begin to close in response to the heat, conserving water (Abhishree et al, 2024). This phenomenon was more evident in the high-wax genotypes, where reduced cuticular transpiration might have led to greater leaf surface heating compared with low-wax genotypes. Low-wax genotypes, by maintaining a higher level of cuticular transpiration, may have benefitted from enhanced evaporative cooling at the leaf surface. These findings suggest that at night, E_{wax} may be beneficial for reducing water loss under stressful conditions, such as high temperatures, by minimizing transpiration. However, under normal well-watered conditions, the presence of high E_{wax} could cause the plant to lose more water during the night due to open stomata, indicating a trade-off between water conservation and the ability to cool the canopy.

Impact of Seasonal and Environmental Factors

The increase in epicuticular wax content during the warmer summer of 2024, compared with 2023 (Tables S2 and S3), highlights the significant role that environmental conditions, particularly temperature, play in modulating plant physiological traits. The nearly doubled E_{wax} content likely reflects a stress-adaptive response aimed at reducing transpiration under more extreme conditions. Elevated temperatures can stimulate the synthesis of epicuticular wax, as plants often enhance wax accumulation in response to heat stress to mitigate water loss and protect against overheating (Medeiros et al, 2017). The wax layer functions as an insulating barrier, helping to maintain cooler leaf surfaces. However, high wax genotypes did not exhibit a proportionate reduction in biomass, indicating that wax alone may not be sufficient to counteract high-temperature conditions (Sreeman et al, 2018). Other protective mechanisms likely upregulate during heat stress, playing a dominant role in reducing heat damage (Chaffai et al, 2024). Although the percent increase in E_{wax} was similar across genotypes, suggesting its importance under high-temperature conditions, the reductions in biomass varied significantly. For instance, among the low wax genotypes, one demonstrated a lower reduction in biomass while another showed a higher reduction (Table S2a), indicating that while epicuticular wax can reduce water loss and serve as a barrier to heat, other mechanisms associated with total radiation intercepted and its utilization efficiency for photosynthesis would determine a genotype's efficiency in coping with stress. Among the tested lines, the genotype IRG 349, which had high leaf wax content and the least reduction in biomass, stands out as a potential candidate for further exploration of heat tolerance mechanisms. This suggests that a multifaceted approach, considering both wax accumulation and other physiological traits, is essential for understanding and improving water use efficiency and heat resilience in plants.

The positive relationship between epicuticular wax (E_{wax}) and nocturnal transpiration suggests a potential trade-off between cuticular resistance and nocturnal water loss. However, at elevated temperatures, genotypes with higher wax content exhibited a smaller increase in nocturnal transpiration, implying a protective role of wax, potentially through modulating stomatal regulation due to increased leaf surface temperatures. This study based on measured parameters, lays a foundation for future research to further dissect the genetic and environmental factors controlling nocturnal transpiration and its modulation by wax deposition. Exploring these mechanisms would provide valuable insights into strategies for regulating nocturnal water loss and improving heat tolerance, crop water budgeting and water use efficiency in crops.

METHODS

Plant materials and growth conditions

The Experiment 1 conducted in Monsoon season of 2023 involved screening 100 diverse rice germplasm lines from the 3K-IRRI collection for their leaf epicuticular wax (E_{wax}) content in open field conditions (Table S1). Based on the screening of E_{wax}, three contrasting groups were established: high wax (IRG 066 and IRG 349), medium wax (IRG 355 and IRG 263), and low wax (IRG 277 and IRG 335) were selected for Experiment 2. These genotypes were then used for understanding nocturnal transpiration and experiment was conducted during summer season of 2024 in the novel drought-simulator phenomics facility in Bengaluru, India (12º58′ N, 77º35′ E). Each genotype was grown in 25 L capacity pots filled with a mixture of red sandy loam soil and farmyard manure (3:1). Such pots were maintained in three replications. Direct sowing was carried out, and thinning was performed 21 d after sowing to retain one plant per pot. The recommended dose of fertilizers and prophylactic measures were applied to ensure healthy and uniform plant growth.

Measurement of morpho-physiological parameters

At harvest, various measurements were taken, including plant height, tiller count, leaf width and specific leaf area. The specific leaf area was calculated as the ratio of known fresh leaf area to its corresponding dry weight (Vijayaraghavareddy et al, 2020b). The total leaf area (TLA) per pot was calculated by multiplying the final dry leaf biomass by the specific leaf area, which was determined from a representative leaf sub-sample (Vijayaraghavareddy et al, 2020b). At harvest, the total above-ground biomass was measured, and the plants were separated into leaves, stems, and panicles. The panicles were then threshed, and the total weight of filled seeds was recorded to determine the overall seed yield. The remaining plant parts were dried at 65 ºC for five days to obtain the total above-ground biomass (g/pot). Finally, the seed yield and above-ground biomass were combined to determine the total dry matter (TDM).

Measurement of Epicuticular wax content:

In both the experiments, the cuticular wax content was quantified using a colorimetric method, which relies on the colour change induced by the reaction of wax with acidic potassium dichromate ($K_2Cr_2O_7$), as outlined by Vijayaraghavareddy et al (2022). A fresh leaf sample at the flowering stage was immersed in 15 mL of redistilled chloroform for 5 s. The length and width of the immersed leaf were measured using a ruler to determine the wax content per unit leaf area. The chloroform extract was then heated at 75 ºC until the chloroform odour completely dissipated. Subsequently, $K_2Cr_2O_7$ reagent was added, and the test tubes were maintained at 65 ºC for 30 min. After cooling, 12 mL of deionized water was added. The absorbance of the samples was measured at 590 nm using a spectrophotometer. A standard curve was generated using carnauba wax of varying concentrations (Sigma, St. Louis, Missouri, USA), as described by Samdur et al (2003). The total cuticular wax content was expressed in micrograms per unit leaf area (µg/cm).

Scanning electron microscope imaging

For the identified contrasts (experiment 2), scanning electron microscope (SEM) imaging was performed on young fully opened leaf samples collected at flowering stage, during periods of active sunlight. The samples were fixed in a sterile 0.1 mol/L phosphate buffer solution (PBS) containing 3.7% formaldehyde

(Priks et al, 2020). Vacuum was applied for 1–2 h until the tissues settled at the bottom, followed by overnight incubation at 4 ºC. After two washes with PBS, the samples underwent dehydration using a series of ethyl alcohol (EtOH) concentrations of 40%, 50%, 60%, 70%, 90%, and 96% for 30 min and of 99.5% for overnight each at room temperature. The samples were then subjected to critical point drying (CPD) using CO_2 (Quorum K850, QUORUM, East Sussex, UK). The dried samples were mounted on stubs, coated with a 20 nm layer of gold (Quorum SC7620, QUORUM), and examined with a digital SEM (Carl Zeiss EVO-18, Zeiss, Oberkochen, Germany).

A novel phenomics facility for precise irrigation and transpiration measurement

Second experiment was conducted in the novel drought-simulator phenomics facility employs mini-lysimeters based on gravimetric principles (Vijayaraghavareddy et al, 2020b; Lekshmy et al, 2021), capable of measuring weights with a precision of 5 g. Weight loss due to transpiration is monitored by specialized software, which triggers solenoid valves to automatically irrigate whenever there is 120 g weight loss in the pots. This system maintains consistent soil moisture at a set Field Capacity (FC), regardless of leaf area and transpiration rate variations. The software records hourly weight loss throughout the crop period, allowing real-time transpiration measurements. This facility is in open field conditions, where we can check plant response to fluctuating natural weather conditions, hence it depicts the actual plant response compared with controlled conditions.

To quantify nocturnal transpiration, we measured the cumulative water transpired per hour during the night (CWT_{night}) by subtracting the nighttime evaporation from the total evapotranspiration recorded during the night hours. Evaporation was measured using five empty containers placed under identical environmental conditions as the experimental plants. Plastic beads were added to these containers to minimize soil surface evaporation. The difference between the total evapotranspiration and the measured evaporation values represented the actual transpired water.

To analyse how transpiration varied across different stages of the night, CWT_{night} was categorized into three distinct periods: early night ($CWT_{earlynight}$, 18:00–00:00 h), late night ($CWT_{latenight}$, 00:00–06:00 h), and predawn (PredawnCWT, 04:00–06:00 h). These periods were chosen to assess the temporal variation in transpiration and focus on the critical early morning hours just before dawn. The early night period corresponds to the initial phase of nocturnal transpiration, while the late-night phase captures the latter part of the night, and the predawn period specifically examines transpiration dynamics in the two hours leading up to sunrise.

To account for differences in plant size, MTR for each period was calculated by normalizing the respective CWT values to the leaf area of the plant. This normalization ensured that the transpiration measurements were independent of leaf area, allowing for accurate comparisons between plants of different sizes.

Meteorological data and transpiration at high temperatures

To study changes in transpiration under natural conditions with varying nighttime temperatures during April 2024, we collected meteorological data, including minimum temperature and maximum relative humidity, from the Meteorology Department at the University of Agricultural Sciences, Bangalore. Our focus was on analyzing transpiration changes corresponding to a 0.2 ºC increment in minimum temperature, starting from the lowest recorded temperature for the month, which was 20 ºC.

To ensure robust data selection, days with similar minimum temperatures were grouped together. For each temperature increment, we prioritized selecting two days that were closest in terms of their minimum temperature values (Fig. S3). However, for certain temperature intervals where data was available for only one day, that single day's data was utilized for analysis. The selected days for each temperature category are as follows: 20.0 ºC: April 6 and April 12; 20.2 ºC: April 11 and April 14; 20.4 ºC: April 1 and April 10; 20.6 ºC: April 13 and April 22; 20.8 ºC: April 5; 21.0 ºC: April 8 and April 9; 21.2 ºC: April 23 and April 27; 21.4 ºC: April 24 and April 29; 21.6 ºC: April 4; and 21.8 ºC: April 7.

For days with two records showing the same minimum temperature, we calculated the mean CWT value to represent the temperature category. For temperature categories with data available for only one day, the single day’s CWT value was directly used in the analysis. The minimum temperature was chosen for this study as it reflects nighttime conditions, specifically warmer nights, which are critical for understanding transpiration patterns under heat stress. The percentage increase in CWT during the night was calculated for every 0.2 ºC increase in temperature compared with the baseline temperature of 20 ºC.

Statistical analysis

One-way analysis of variance (ANOVA) was performed using GraphPad Prism 8.0.1. The least significant difference for each parameter was employed to assess significance levels. To examine the relationship between different parameters, correlation and regression analyses were carried out using ‘corrplot’ and ‘ggplot2’ packages, respectively, in R studio version 4.4.1.

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SUPPLEMENTARY DATA

The following materials are available in the online version of this article at http://www.sciencedirect.com/journal/rice-science; http://ricescience.org.

Fig. S1. Images from scanning electron microscopy showing differences in epicuticular was content (E_{wax}) content.

Fig. S2. Correlation analysis between various parameters.

Fig. S3. Weather parameters.

Fig. S4. Variability in hourly transpiration among rice genotypes with differing epicuticular wax (E_{wax}) content at varying minimum night temperatures.

Table S1.List of genotypes and their epicuticular wax content (E_{wax}).

Table S2. Variation in total dry matter (TDM) among rice genotypes with differing E_{wax}.

Table S3. Slope of the curve calculated from rate of increase in transpiration per 0.2 ºC increase in temperature compared with 20.0 ºC.

REFERENCES

Bahuguna R N, Chaturvedi A K, Pal M, et al. 2022. Carbon dioxide responsiveness mitigates rice yield loss under high night temperature. *Plant Physiol*, **188**(1): 285-300.

Berkeley Earth. 2023. Global temperature report for 2023. https://berkeleyearth.org/global-temperature-report-for-2023/.

Blum A. 1975. Effect of the bm gene on epicuticular wax deposition and the spectral characteristics of sorghum leaves. *SABRAO J*, **7**(1): 45-52.

Caird M A, Richards J H, Donovan L A. 2007. Nighttime stomatal conductance and transpiration in C_3 and C_4 plants. *Plant Physiol*, **143**(1): 4-10.

Carvalho H D R, Heilman J L, McInnes K J, et al. 2020. Epicuticular wax and its effect on canopy temperature and water use of Sorghum. *Agric For Meteor*, **284**: 107893.

Chaffai R, Ganesan M, Cherif A. 2024. Regulatory mechanisms in plant response to cold stress. *In*: Plant Adaptation to Abiotic Stress: From Signaling Pathways and Microbiomes to Molecular Mechanisms. Singapore: Springer: 49-59.

Coupel-Ledru A, Lebon E, Christophe A, et al. 2016. Reduced nighttime transpiration is a relevant breeding target for high water-use efficiency in grapevine. *Proc Natl Acad Sci USA*, **113**(32): 8963-8968.

Dawson T E, Burgess S S O, Tu K P, et al. 2007. Nighttime transpiration in woody plants from contrasting ecosystems. *Tree Physiol*, **27**(4): 561-575.

de Dios V R, Roy J, Ferrio J P, et al. 2015. Processes driving nocturnal transpiration and implications for estimating land evapotranspiration. *Sci Rep*, **5**: 10975.

Desai J S, Lawas L M F, Valente A M, et al. 2021. Warm nights disrupt transcriptome rhythms in field-grown rice panicles. *Proc Natl Acad Sci USA*, **118**(25): e2025899118.

Fricke W. 2019. Night-time transpiration-favouring growth? *Trends Plant Sci*, **24**(4): 311-317.

Fricke W. 2020. Energy costs of salinity tolerance in crop plants: Night-time transpiration and growth. *New Phytol*, **225**(3): 1152-1165.

Holmes M G, Keiller D R. 2002. Effects of pubescence and waxes on the reflectance of leaves in the ultraviolet and photosynthetic wavebands: A comparison of a range of species. *Plant Cell Environ*, **25**(1): 85-93.

Impa S M, Raju B, Hein N T, et al. 2021. High night temperature effects on wheat and rice: Current status and way forward. *Plant Cell Environ*, **44**(7): 2049-2065.

Jarin A S, Islam M M, Rahat A, et al. 2024. Drought stress tolerance in rice: Physiological and biochemical insights. *Int J Plant Biol*, **15**(3): 692-718.

Koch K, Ensikat H J. 2008. The hydrophobic coatings of plant surfaces: Epicuticular wax crystals and their morphologies, crystallinity and molecular self-assembly. *Micron*, **39**(7): 759-772.

Lekshmy V S, Vijayaraghavareddy P, Nagashree A N, et al. 2021. Induction of acquired tolerance through gradual progression of drought is the key for maintenance of spikelet fertility and yield in rice under semi-irrigated aerobic conditions. *Front Plant Sci*, **11**: 632919.

Lewandowska M, Keyl A, Feussner I. 2020. Wax biosynthesis in response to danger: Its regulation upon abiotic and biotic stress. *New Phytol*, **227**(3): 698-713.

Medeiros C D, Falcão H M, Almeida-Cortez J, et al. 2017. Leaf epicuticular wax content changes under different rainfall regimes, and its removal affects the leaf chlorophyll content and gas exchanges of *Aspidosperma pyrifolium* in a seasonally dry tropical forest. *S Afr N J Bot*, **111**: 267-274.

Nagaraju S, Ramesh M, Mujjassim N E, et al. 2024. Rice night-time thirst: Genotype nutrient needs reflected in nocturnal transpiration. *Rhizosphere*, **32**: 100956.

NASA. 2021. Global climate change impact on crops expected within 10 years. https://climate.nasa.gov/news.

NASA. 2023. NASA analysis confirms 2023 as warmest year on record. https://www.nasa.gov/news-release/nasa-analysis-confirms-2023-as-warmest-year-on-record/.

Nievola C C, Carvalho C P, Carvalho V, et al. 2017. Rapid responses of plants to temperature changes. *Temperature*, **4**(4): 371-405.

NOAA (National Oceanic and Atmospheric Administration). 2023. 2023 was the world’s warmest year on record, by far. https://www.noaa.gov/news/2023-was-worlds-warmest-year-on-record-by-far.

Ohtaka K, Yoshida A, Kakei Y, et al. 2020. Difference between day and night temperatures affects stem elongation in tomato (*Solanum lycopersicum*) seedlings via regulation of gibberellin and auxin synthesis. *Front Plant Sci*, **11**: 577235.

Priks H, Butelmann T, Illarionov A, et al. 2020. Physical confinement impacts cellular phenotypes within living materials. *ACS Appl Bio Mater*, **3**(7): 4273-4281.

Rahman T, Shao M X, Pahari S, et al. 2021. Dissecting the roles of cuticular wax in plant resistance to shoot dehydration and low-temperature stress in *Arabidopsis*. *Int J Mol Sci*, **22**(4): 1554.

Ramachandra A, Vijayaraghavareddy P, Purushothama C, et al. 2024. Decoding stomatal characteristics regulating water use efficiency at leaf and plant scales in rice genotypes. *Planta*, **260**(3): 56.

Richards R A, Rawson H M, Johnson D A. 1986. Glaucousness in wheat: Its development and effect on water-use efficiency, gas exchange and photosynthetic tissue temperatures. *Funct Plant Biol*, **13**(4): 465-473.

Riederer M, Schreiber L. 2001. Protecting against water loss: Analysis of the barrier properties of plant cuticles. *J Exp Bot*, **52**: 2023-2032.

Sadok W, Jagadish S V K. 2020. The hidden costs of nighttime warming on yields. *Trends Plant Sci*, **25**(7): 644-651.

Sadok W, Lopez J R, Smith K P. 2021. Transpiration increases under high-temperature stress: Potential mechanisms, trade-offs and prospects for crop resilience in a warming world. *Plant Cell Environ*, **44**(7): 2102-2116.

Samdur M Y, Manivel P, Jain V K, et al. 2003. Genotypic differences and water-deficit induced enhancement in epicuticular wax load in peanut. *Crop Sci*, **43**(4): 1294-1299.

Shellakkutti N, Thangamani P D, Suresh K, et al. 2022. Cuticular transpiration is not affected by enhanced wax and cutin amounts in response to osmotic stress in barley. *Physiol Plant*, **174**(4): e13735.

Shepherd T, Wynne Griffiths D. 2006. The effects of stress on plant cuticular waxes. *New Phytol*, **171**(3): 469-499.

Sreeman S M, Vijayaraghavareddy P, Sreevathsa R, et al. 2018. Introgression of physiological traits for a comprehensive improvement of drought adaptation in crop plants. *Front Chem*, **6**: 92.

Vijayaraghavareddy P, Vemanna R S, Yin X Y, et al. 2020a. Acquired traits contribute more to drought tolerance in wheat than in rice. *Plant Phenomics*, **2020**: 5905371.

Vijayaraghavareddy P, Yin X Y, Struik P C, et al. 2020b. Responses of lowland, upland and aerobic rice genotypes to water limitation during different phases. *Rice Sci*, **27**(4): 345-354.

Vijayaraghavareddy P, Lekshmy S V, Struik P C, et al. 2022. Production and scavenging of reactive oxygen species confer to differential sensitivity of rice and wheat to drought stress. *Crop Environ*, **1**(1): 15-23.

Wahid A, Gelani S, Ashraf M, et al. 2007. Heat tolerance in plants: An overview. *Environ Exp Bot*, **61**(3): 199-223.

Weerakoon W M W, Maruyama A, Ohba K. 2008. Impact of humidity on temperature-induced grain sterility in rice (*Oryza sativa* L). *J Agron Crop Sci*, **194**(2): 135-140.

Yan Y L, Ryu Y, Dechant B, et al. 2023. Dark respiration explains nocturnal stomatal conductance in rice regardless of drought and nutrient stress. *Plant Cell Environ*, **46**(12): 3748-3759.

Yang Z Q, Jiang Y H, Qiu R J, et al. 2023. Heat stress decreased transpiration but increased evapotranspiration in *Gerbera*. *Front Plant Sci*, **14**: 1119076.

Yi Y, Yano K. 2023. Nocturnal versus diurnal transpiration in rice plants: Analysis of five genotypes grown under different atmospheric CO_2 and soil moisture conditions. *Agric Water Manag*, **286**: 108397.

Zhao C, Liu B, Piao S L, et al. 2017. Temperature increase reduces global yields of major crops in four independent estimates. *Proc Natl Acad Sci U S A*, **114**(35): 9326-9331.