

NONDESTRUCTIVE OPTICAL SPECTROSCOPY IN PLANT STRESS RESEARCH: CIRCADIAN RHYTHM AS A DIAGNOSTIC MARKER

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ABSTRACT

In the context of environmental changes and the increasing demand for sustainable agricultural practices, real-time monitoring of plant health is increasingly important. This work provides an overview of the development and application of nondestructive optical spectroscopy for early detection of stress across a wide range of plant species. The approach combines high-resolution time tracking of leaf transmission with circadian rhythm analysis, allowing the identification of subtle physiological changes that precede visible stress symptoms.

This work presents results from several experimental studies, including hydroponically grown herbs, forest species, aquatic plants, ornamentals, and agricultural crops. The methodology enables the early detection of stress caused by nutritional deficiencies, pathogenic infections, and sudden changes in light intensity. Integration of the 640 nm and 665 nm spectrum bands significantly improved system sensitivity, allowing precise characterisation of metabolic responses. These advances are supported by comprehensive metrological validation, which ensures the repeatability and robustness of the data under experimental conditions.

The lecture highlights circadian rhythm not only as a fundamental biological process, but also as a new diagnostic marker of the physiological state of the plant. Through a variety of case studies and practical applications, we demonstrate how this optical platform contributes to improving understanding the response of plants to stress and offers new perspectives in plant science, forest monitoring, and precision agriculture.

Key words: Nondestructive spectroscopy, circadian rhythm, plant stress detection, real-time plant monitoring, leaf transmittance.

INTRODUCTION

Modern plant cultivation stands at the crossroads of profound societal and technological transformation. The need to ensure global food security, adapt to climate change, and minimise environmental impact makes the prompt and efficient detection and management of plant stress a critical priority. According to the Food and Agriculture Organisation (FAO) and recent international reports, up to 40% of potential crop yield is lost annually due to environmental stress factors such as drought, salinity, pathogens, and nutrient imbalance, directly impacting economies, societies, and the stability of food systems (Kumar et al., 2022). These challenges underscore the importance of innovations that bridge advanced science with practical application, empowering both growers and decision-makers.

Thus, early detection of plant stress is not only a scientific or agronomic priority but also a key component of sustainable development, environmental protection, and societal resilience. Effective and precise monitoring of plant physiological status can reduce agricultural losses,

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improve yield quality, optimise the use of chemical input, and support data-driven decision making all fundamental goals in the transition to smart and sustainable agriculture.

Traditional plant health monitoring has typically relied on destructive, time-consuming, and often subjective methods. These approaches involve physicochemical analyses of plant tissues, which require laborious sample preparation and often result in damage to the plant material (Kvet et al., 1971). Moreover, such methods provide only fragmented and retrospective insights, often missing the early stages of stress response when intervention is most effective. Given that the general state is closely related to the intensity of photosynthetic processes in its leaves, there is a critical need for a continuous and nondestructive monitoring of these key physiological indicators (Gitelson et al., 2023).

Nondestructive optical methods (NOM) have recently emerged as transformative tools in this regard. These techniques allow for real-time in vivo assessment of plant metabolism and stress, preserving plant integrity, and enabling the detection of subtle physiological changes before visible symptoms arise. At the Faculty of Physics, University of Belgrade, our group has developed and validated an innovative NOM system that continuously monitors plant health and metabolic processes by measuring optical transmission and reflection primarily at wavelengths relevant for chlorophyll absorption (e.g., 665 nm and more recently, 640 nm) (Kasalica et al., 2021; Miletic et al., 2022b; Miletic et al., 2023a; Miletic et al., 2023b; Miletic et al., 2023c; Miletic et al., 2023d; Miletic et al., 2024a; Miletic et al., 2024b, Ristic et al., 2023, Ristic et al., 2024).

An identifying feature of this approach is the analysis of circadian rhythm patterns in the optical properties. Since photosynthetic activity within leaves is tightly coupled with overall plant health, disruptions or modifications in circadian patterns manifested as changes in the amplitude, frequency, or phase of optical signals can serve as sensitive early markers of diverse stress conditions. The design of the system, with multiple independent measurement points and flexible leaf holders, ensures statistically robust and reliable data, accommodating natural leaf movement and environmental variability.

Over recent years, this NOM technology has been successfully applied to detect and characterise a wide range of plant stresses, including: light intensity stress, nutritional deficiencies, water scarcity/drought stress, pathogen infections, herbicide exposure, etc. (Miletic et al., 2022a; Veljovi Jovanovi et al., 2023; Miletic et al., 2023b; Madzarevic et al., 2023; Mladenovic et al., 2023; Komazec et al., 2024).

The efficacy and sensitivity of the NOM system have been benchmarked against traditional parameters, such as pigment content, dry weight, and Photosystem II (PSII) efficiency, with optical methods often revealing changes well before conventional indices (Kasalica et al., 2021; Miletic et al., 2022a; Veljović Jovanović et al., 2023, Ristic et al., 2024)

Beyond the scientific and technical achievements, the social impact of this methodology is substantial. By providing an accessible, rapid, and precise means of monitoring plant health, NOM facilitates the digitalisation and smart management of agriculture optimising resource use, reducing environmental impact, and supporting the resilience of food systems. This is especially relevant as societies face the compounded pressures of climate change, resource limitations, and the need for a sustainable intensification of agricultural production (FAO, 2022).

In summary, the development and application of nondestructive optical monitoring systems mark a significant advance in plant science and technology, opening paths for more efficient, sustainable, and socially responsible agriculture.

MATERIALS AND METHODS

Measurement System and Optical Setup

The metrological foundation of this system is built on precise measurement principles and a robust experimental setup, designed for high sensitivity and statistical reliability in tracking circadian rhythms as key indicators of metabolic activity and stress (Kasalica et al., 2021).

The core of the NOM developed at the Faculty of Physics, University of Belgrade, is a custom designed multichannel system for real-time monitoring of the optical properties of the plant leaf (Figure 1). The apparatus consists of 20 independent measurement channels (Figure 2), each

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accommodating a single leaf attached via a transparent adjustable holder that allows for five degrees of mechanical freedom, allowing the system to follow the natural movement and growth of the plant leaf. Each measurement channel uses red LED light sources (primarily 665 nm, with recent upgrades including 640 nm for enhanced photosynthetic specificity), coupled via optical fibres to the plant leaf. Transmitted and reflected light is collected by dedicated fibres and detected by photodiodes within a thermostated, shielded compartment. Signals are filtered at precise wavelengths and recorded at 15-minute intervals using automated data acquisition and custom software. Calibration is routinely performed with certified neutral density filters and standard reflective targets to ensure data comparability across all channels (Kasalica et al., 2021; Miletic et al., 2024b).

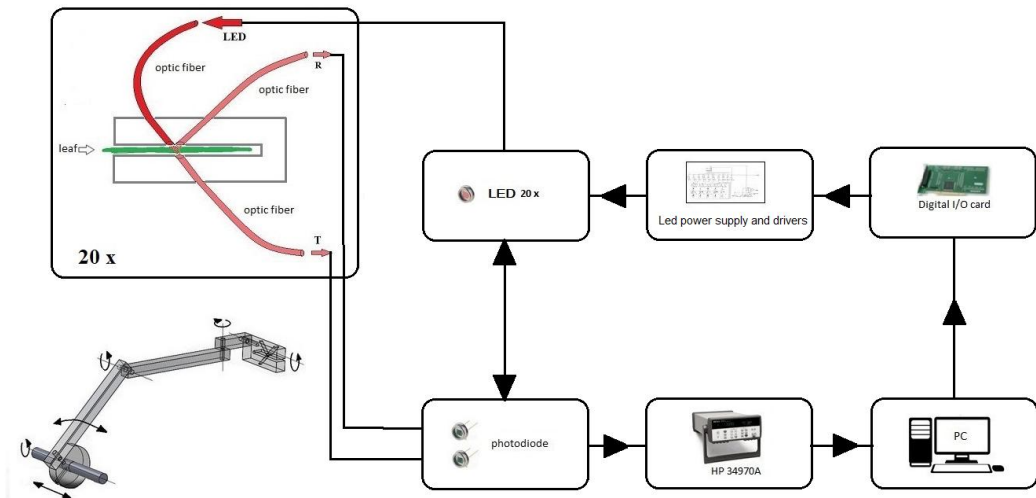


Figure 1. NOM block diagram (Kasalica et al., 2021)

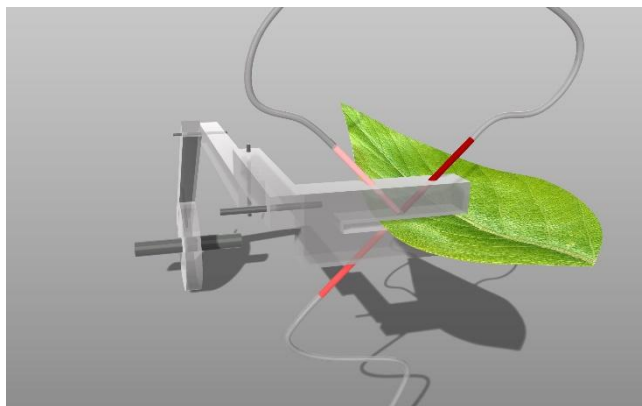


Figure 2. Optical-fibre-integrated acrylic leaf holders

Plant Material and Stress Treatments

Experiments are conducted in controlled indoor environments or growth chambers with regulated temperature, photoperiod, humidity, and illumination. Plants are cultivated hydroponically or in soil, depending on the specific experimental goal. Environmental parameters

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(e.g., temperature, humidity, light intensity/photosynthetic photon flux density – PPFD) are regularly monitored and maintained to ensure reproducibility and minimise external variability.

Various plant species have been studied using this system, including *Ocimum basilicum*, *Vriesea carinata*, *Pelargonium zonale*, and *Castanea sativa*, among others. For stress treatments, experimental groups are subjected to specific stressors:

- Nutritional deficiency: Nutrient solutions lacking key elements (N, Mg, P, Fe) are prepared, and control plants are maintained in complete nutrient solutions (Miletic et al., 2022a).
- Water scarcity: Watering is stopped for a defined period to induce drought stress (Miletic et al., 2023c).
- Light-intensity stress: Plants are exposed to varying light intensities, controlled by dimmable LED panels, with measurements performed across a range of PPFD values (Kasalica et al., 2021; Veljović Jovanović et al., 2023).
- Pathogen infection: Various plants are inoculated with pathogens, such as *Phytophthora plurivora*, using established flood and inoculation protocols (Miletic et al., 2023b).
- Herbicide application: Some experiments include treatment with commonly used herbicides, with subsequent monitoring (Miletic et al., 2023d).

Data Acquisition and Analysis

Optical transmission and reflection coefficients are recorded for each channel over time, yielding time-resolved profiles suitable for circadian rhythm analysis. Parameters such as amplitude, period, and deviations from the baseline are extracted to serve as early stress indicators. For method validation, traditional physiological and biochemical measurements (e.g., pigment content, dry weight, PSII efficiency) are conducted in parallel, as detailed in previous studies (Kasalica et al., 2021; Miletic et al., 2022a; Veljović Jovanović et al., 2023; Ristic et al., 2024).

Calibration and System Validation

Calibration procedures account for interchannel variability, LED emission characteristics, and optical path differences, using neutral-density filters and standard diffusive targets. Systematic validation is performed by comparing the NOM data with conventional methods, ensuring the accuracy and sensitivity of the approach. Further technical and methodological details are available in our referenced publications (Kasalica et al., 2021; Miletic et al., 2022a; Veljović Jovanović et al., 2023; Ristic et al., 2024).

RESULTS

The NOM system enabled continuous, real-time monitoring of plant responses under various stress conditions. Alterations in the circadian rhythm of optical transmission were observed as early and sensitive indicators of stress, frequently preceding visible symptoms or changes detected by conventional methods.

Detection of nutrient deficit stress

Continuous monitoring of leaf optical properties revealed that plants subjected to nutrient deficiency exhibit characteristic alterations in the circadian rhythm of transmission well before any visible symptoms are observed. In hydroponically grown *Ocimum basilicum*, significant deviations from the control circadian pattern manifested as reduced amplitude and baseline shifts were detected as early as 2 to 4 days before the onset of leaf chlorosis or measurable pigment loss. The observed optical changes arise from the physiological consequences of nutrient deprivation, primarily chlorophyll degradation, altered pigment composition, and structural rearrangements within mesophyll tissue, which jointly modify the absorption and scattering of red light through the leaf. These processes reduce the effective optical density of the leaf, resulting in modified transmission amplitude and rhythm stability. The same mechanism was previously confirmed through parallel measurements of chlorophyll content and PSII efficiency, which showed a strong correlation with optical response (Miletic et al., 2022a). Restoration of missing nutrients resulted in

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recovery of normal circadian oscillations, confirming both the sensitivity and reversibility of the system (Madzarevic et al., 2023). This recovery reflects the reestablishment of photosynthetic balance and pigment synthesis, further validating the circadian optical signal as a reliable indicator of metabolic adjustment. Figure 3 illustrates the typical disruption of the circadian rhythm due to nutrient deficiency.

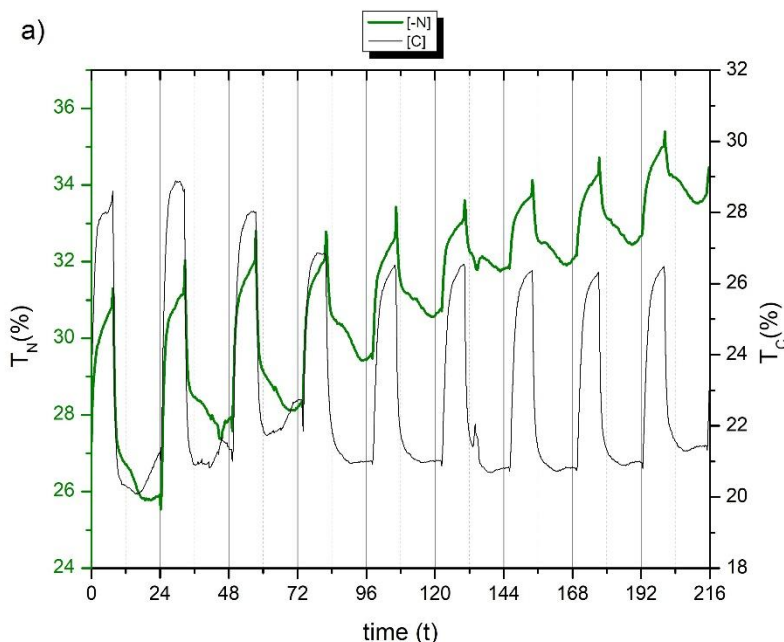


Figure 3. Comparative circadian rhythm profiles of *Ocimum basilicum* under full nutrient supply and nitrogen deficiency (Miletic et al., 2022a)

Light Intensity Stress and Photometabolic Shifts

When exposed to excessive light, species such as *Vriesea carinata* and *Pelargonium zonale* demonstrated distinct changes in the amplitude and phase of circadian rhythm profiles. These changes are primarily associated with photoinhibition effects and dynamic photoprotective responses that occur when light absorption temporarily exceeds the capacity of the photosynthetic electron transport chain. Increased photon flux density induces adjustments in chloroplast positioning and activation of non-photochemical quenching mechanisms, both of which alter light scattering and absorption properties within the leaf (Veljović Jovanović et al., 2023). The NOM system successfully tracked the transitions between C3 photosynthesis and CAM (Crassulacean Acid Metabolism) in *V. carinata*, with high light intensities that cause an increase in amplitude and a change in the timing of daily maxima and minima (Figure 4). This transition reflects a physiological shift in carbon fixation and stomatal behaviour, where nocturnal CO₂ uptake and daytime decarboxylation modify the temporal pattern of photosynthetic activity. As CAM metabolism becomes dominant, light transmission increases during the night due to stomatal opening and water retention, while daytime transmittance decreases as the stomata closes and internal CO₂ recycling prevails. These metabolic adjustments produce characteristic optical phase shifts and modulation that the NOM system captures in real time. CAM species open their stomata predominantly at night and often exhibit circadian phase shifts compared to C3, which is relevant for interpreting transmittance rhythms (Ristic et al., 2024).

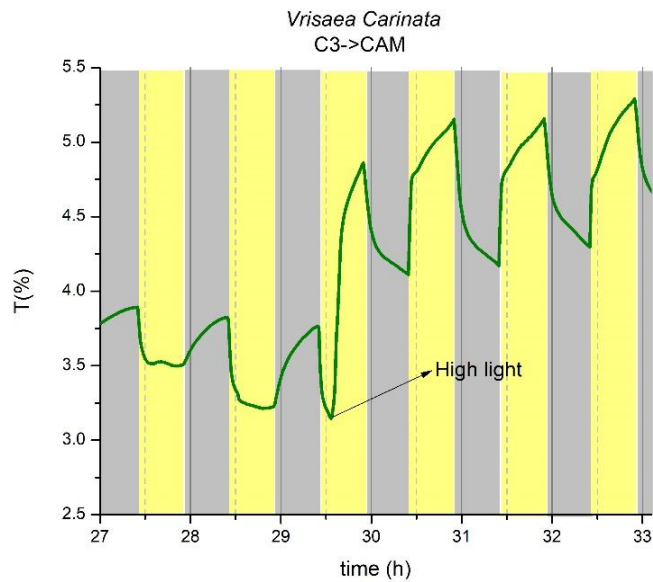


Figure 4. Circadian rhythm of *Vriesaea carinata* under changing light intensity (Ristic et al., 2024)

Furthermore, the recently integrated 640 nm channel enabled even earlier detection of stress-induced changes, as shown in comparative results for the 665 nm and 640 nm channels (Miletic et al., 2024b).

Figure 5 presents representative circadian profiles under varying light regimes, highlighting differences between detection wavelengths.

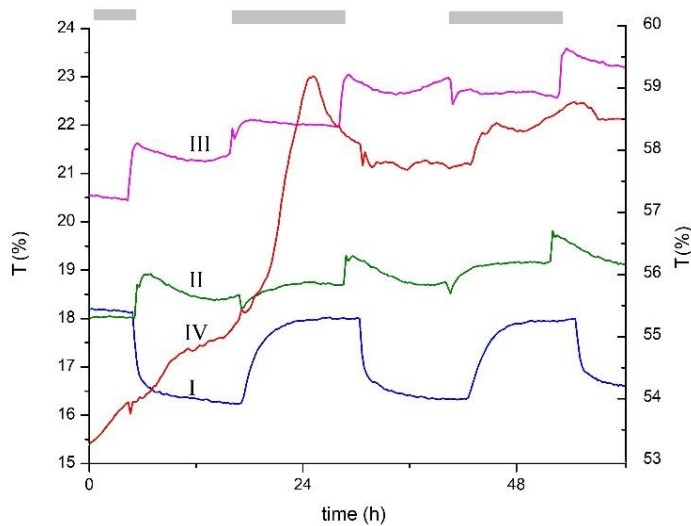


Figure 5. Circadian rhythm of the *P. zonale* under changing light intensity (Veljović Jovanović et al., 2023)

Drought (Water Scarcity) Stress

Under water deficiency conditions, significant disturbances in circadian rhythm were observed several days before the appearance of visual signs of wilting or necrosis. In *Ocimum Basilicum*, the amplitude of the circadian signal decreased and the baseline changed as the availability of water decreased. These optical alterations primarily result from reduced leaf turgor and intracellular water content, which influence refractive gradients within the mesophyll and modify the balance between transmitted and scattered light. As dehydration progresses, stomatal closure limits CO₂ uptake and photosynthetic electron flow, reducing chlorophyll excitation and light absorption efficiency. The combined effects lead to a gradual decrease in transmission amplitude and a change in circadian baseline, both of which were consistently detected by the NOM system prior to visible stress symptoms (Miletić et al., 2023c). In particular, the system could reliably differentiate between mild and severe drought stress states based on rhythm characteristics. Figure 6 shows the temporal evolution of the transmission coefficient during prolonged drought stress.

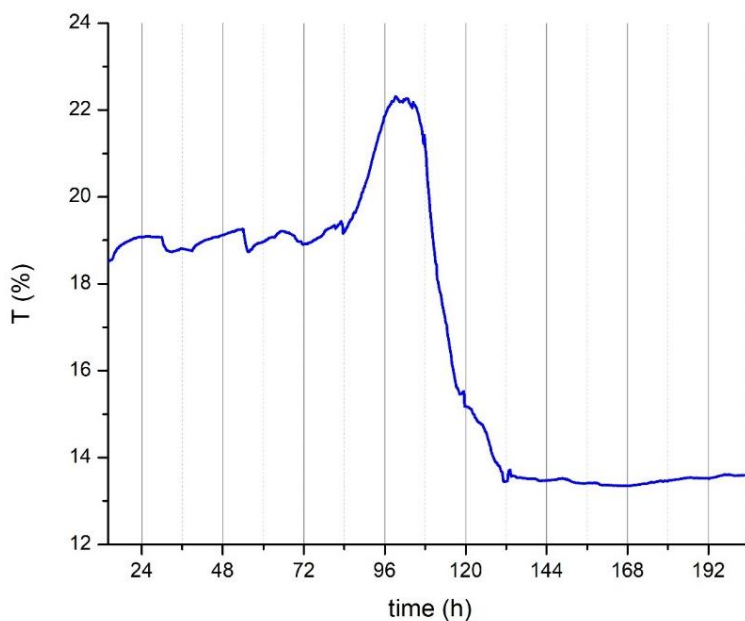


Figure 6. Circadian rhythm of drought-stressed *Ocimum Basilicum* (Miletić et al., 2023c)

Pathogen Infection

Following inoculation with *Phytophthora plurivora*, *Castanea sativa* leaves exhibited rapid and marked alterations in amplitude and structure (Miletić et al., 2023b). These optical changes consistently preceded the appearance of visible disease symptoms, providing a critical window for early intervention and plant protection. Physiologically, infection disrupts chloroplast integrity and damages mesophyll cell membranes, leading to localised water loss and oxidation of pigments. These structural and biochemical alterations modify the internal light scattering pattern and reduce overall transmittance, which manifests as amplitude damping and irregularities in circadian rhythm (Miletić et al., 2023b). The observed phase shifts are related to impaired photosystem activity and oxidative imbalance, making the optical signal a sensitive indicator of early pathogenic stress.

Figure 7 shows the modification of circadian profiles in infected versus control leaves.

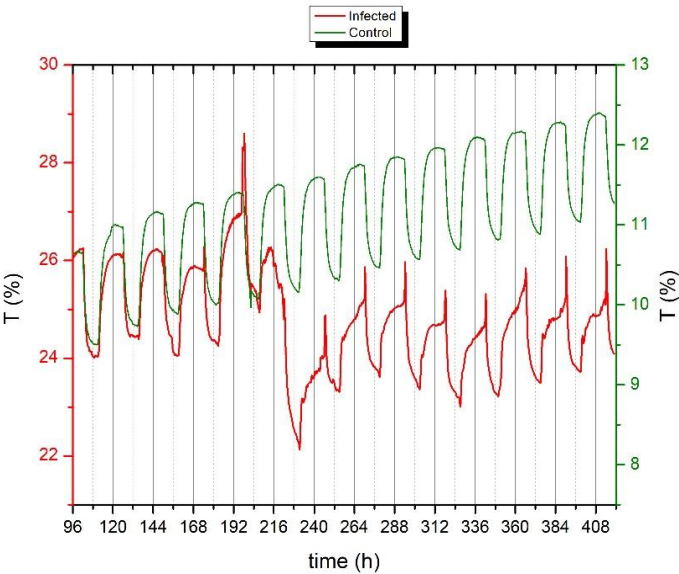


Figure 7. Phase shifts in circadian rhythms of *Castanea sativa* leaves following pathogen infection (Miletic et al., 2023b)

Herbicide-Induced Stress

The application of herbicides led to a pronounced dampening and desynchronisation of the circadian rhythm in exposed plants. Altered periodicity and reduced amplitude were evident within 24 hours after treatment, underscoring the sensitivity of the NOM system to various chemical stressors. These effects arise from the inhibition of photosynthetic electron transport and the degradation of photosynthetic pigments caused by herbicide exposure. The breakdown of chlorophyll biosynthesis reduces the absorption of red light, while membrane damage alters internal scattering pathways within the leaf. As a result, the overall transmittance signal becomes unstable, with a decrease in amplitude and rhythm coherence, providing a clear optical signature of herbicide-induced stress (Miletic et al., 2023d). Figure 8 illustrates typical rhythm alterations after herbicide application.

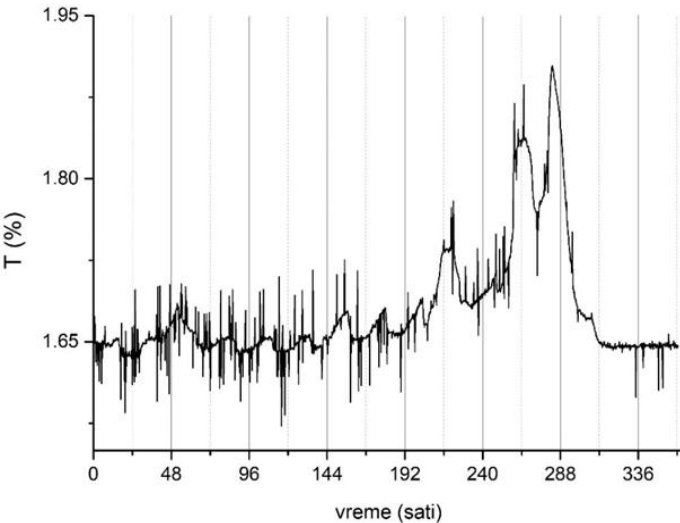


Figure 8. Circadian rhythm of *Bryophyllum daigremontianum* (Miletic et al., 2023d)

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Validation with Conventional Physiological Indices

Across all stressors considered, the NOM system detected changes earlier than traditional physiological measures, such as chlorophyll content, dry weight, and PSII efficiency. Quantitative comparison demonstrated a strong correlation between early NOM signal changes and subsequent alterations in these classical indices, but with NOM offering a substantial advantage in timing and resolution (Kasalica et al., 2021; Miletic et al., 2022a; Veljović Jovanović et al., 2023, Ristic et al., 2024).

DISCUSSION

Early Stress Detection: Scientific and Practical Relevance

The results presented in this study demonstrate the high sensitivity and specificity of the NOM system for the early detection of various plant stresses. The ability to track subtle, stress-induced alterations in circadian rhythms often days before visible or classical physiological symptoms appear represents a major advancement over traditional, destructive, and delayed diagnostic approaches. This provides not only a scientific breakthrough in plant stress physiology, but also a powerful tool for timely intervention in both research and agricultural practice.

Comparison with Conventional Methods and Other Optical Techniques

Unlike standard physiological indices such as chlorophyll content, dry biomass, or PSII efficiency, which often lag behind the actual onset of stress, NOM enables continuous, nondestructive monitoring of metabolic processes. In direct comparison, classical methods typically detect stress at a later stage, when irreversible damage may already have occurred. Incorporating multiple wavelengths, especially the addition of 640 nm channels, further enhances the sensitivity of the method, allowing the discrimination of stress type and even metabolic transitions (e.g. C3 to CAM) in real-time.

Beyond its early-warning capacity, the system demonstrates several distinct advantages over other optical approaches. Its multichannel design ensures robust, parallelised measurements and strong statistical reliability, while its high temporal resolution makes it ideally suited for tracking circadian dynamics. The method has proven versatile across a wide range of plant species and stress conditions, and its performance has been systematically validated against established biochemical and physiological markers, confirming both its accuracy and practical relevance.

Broader Implications for Smart Agriculture and Sustainable Development

Beyond the laboratory, the deployment of NOM technology in field or controlled environment agriculture can contribute significantly to smart, resource efficient, and resilient food production systems. Early and precise stress detection allows targeted interventions, such as optimised irrigation, fertilisation or pest management, to minimise input waste and environmental impact. This aligns with the principles of precision agriculture and supports the broader goals of sustainable development, climate adaptation, and food security.

Furthermore, the nondestructive, continuous, and scalable nature of the method makes it a strong candidate for integration with digital and IoT (Internet of Things)-based monitoring platforms, enabling automated, data-driven crop management and large-scale phenotyping.

Limitations and future directions

Although the current system demonstrates robust performance under controlled experimental conditions, more work is needed to adapt and validate the approach in open field settings, where environmental variability is greater. The development of portable and user-friendly devices, as well as the application of machine learning to automate data interpretation, represent promising avenues for future research.

Additionally, extending the spectral coverage and integrating NOM with other sensing modalities (e.g., hyperspectral imaging, environmental sensors) could further expand its diagnostic power.

CONCLUSION

This work demonstrates that advanced, nondestructive optical monitoring of circadian rhythms provides a highly sensitive and early indicator of plant stress across a range of environmental and physiological challenges. The ability to detect stress-induced metabolic changes days before the appearance of visible symptoms or traditional physiological markers represents a significant advance for both plant science and agricultural practice.

By continuously and nondestructively tracking the optical properties of plant leaves, particularly at wavelengths relevant for photosynthetic processes, the NOM system enables rapid identification, differentiation, and quantification of stress responses. This not only supports more effective research and breeding for stress-tolerant genotypes, but also empowers the implementation of precision agriculture and smart crop management strategies.

The integration of such technologies into agricultural and environmental monitoring systems holds great promise in reducing yield losses, optimising resource use, and supporting the transition to sustainable, resilient, and climate-smart food production. The continued development and deployment of NOM approaches will further strengthen the link between scientific innovation and real-world impact, contributing to the broader goals of sustainable development and food security.

DECLARATIONS OF INTEREST STATEMENT

The authors affirm that there are no conflicts of interest to declare in relation to the research presented in this paper.

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