Multifaceted roles of melatonin: Investigating coffee leaf melatonin concentration as a potential indicator of vegetative growth

Dian Siswanto[1*], *Aldian* Dwi Nanda[1], *Imroatin* Muwahida[1], *Nurul* Istiqomah[2,3], *Aminatun* Munawarti[1], *Retno* Mastuti[1]

[1]Department of Biology, Faculty of Mathematics and Natural Sciences, Brawijaya University, Jl. Veteran Malang 65145, East Java, Indonesia
[2]Doctoral Program, Department of Biology, Faculty of Mathematics and Natural Sciences, Brawijaya University, Malang, 65145, East Java, Indonesia
[3]National Research and Innovation Agency of The Republic of Indonesia (BRIN), Jakarta 10340, Indonesia

Abstract. This study investigates the melatonin profile in the leaves of Liberica (*Coffea liberica*) and Robusta (*Coffea canephora*) coffee plants, assessing its potential as a growth indicator during the vegetative phase. Our research posits that melatonin concentrations vary with leaf age and can be effectively detected through Thin Layer Chromatography (TLC) and Liquid Chromatography-Mass Spectrometry (LC-MS) analysis. Results showed that all leaf samples—young, middle-aged, and old—from both species exhibited melatonin, confirming its role in various physiological processes. LC-MS analysis revealed melatonin concentrations of 0.72% and 0.58% in young and middle-aged Liberica leaves, respectively. In contrast, Robusta leaves showed slightly higher levels in young leaves (0.73%) and lower in middle-aged leaves (0.51%). These findings suggest that melatonin is crucial in promoting growth and stress tolerance, particularly in young leaves. Furthermore, the variations in melatonin concentrations between the two species indicate potential species-specific adaptations related to photosynthesis and environmental resilience. The results underscore the pivotal role of melatonin in the developmental stages of coffee plants and propose its potential as a growth indicator. This finding paves the way for future research to refine and optimize the analytical methods for detection of melatonin, sparking further interest and exploration in this area.

1 Introduction

The development of coffee cultivation in Indonesia dates back to the 17th century, during the era of the *Vereenigde Oostindische Compagnie* (VOC), the largest Dutch trading company. The first coffee introduced to the region was Yemeni Arabica coffee (*Coffea arabica*), which was originally from India. However, Arabica coffee production faced significant challenges due to pest infestations, leading to the introduction of the Liberica coffee variety (*Coffea*

* Corresponding author: diansiswanto@ub.ac.id

liberica) as an alternative [1]. The Dutch imported Liberica coffee in the 19th century due to its resistance to leaf rust (*Hemileia vastatrix*) and adaptability to lowland environments. According to Prasetyo et al. [2], Liberica coffee thrives in high humidity and hot climates, demonstrating resilience to various pests and diseases. Despite these advantages, Liberica coffee is generally considered less productive than other varieties, resulting in its rapid replacement by Robusta coffee (*Coffea canephora*) [3].

Melatonin (N-acetyl-5-methoxytryptamine) is a hormone recognized for its role in enhancing vegetative growth in plants. Naturally, plants synthesize melatonin, increasing production levels in response to stress conditions. This synthesis involves precursors such as tryptamine, 5-hydroxytryptophan, serotonin, and N-acetylserotonin, leading to a wide range of melatonin concentrations, from undetectable to significantly high amounts. Notably, leaves are key organs where melatonin is found [4]. Given the nitrogen-containing compounds involved in melatonin synthesis, it is anticipated that different leaf ages—young, middle-aged, and old—may exhibit varying melatonin concentrations.

First identified in plants in 1995 and referred to as phytomelatonin, this compound plays a crucial role in numerous physiological processes, including defense against environmental stress, seed germination, plant growth, rhizogenesis, aging, photosynthesis, CO_2 uptake, and both primary and secondary metabolism [4]. Additionally, melatonin has been linked to changes in various plant hormones. Despite its significance, its potential as a growth indicator during the vegetative phase, a role that has yet to be fully explored, holds great promise and is an exciting area for future research.

Plant growth requires both macronutrients and micronutrients, with a particularly high demand for nitrogen during the vegetative phase. Melatonin is synthesized in mitochondria and chloroplasts, allowing it to be present in various plant tissues [4]. L-tryptophan, a nitrogen-containing compound, serves as the primary precursor for melatonin biosynthesis. The importance of nitrogen in this process is significant, as leaf development varies across different stages—young, middle-aged, and old—so do the nitrogen requirements, which are believed to influence melatonin concentrations. Thus, measuring melatonin levels in leaves of varying ages is expected to reveal significant correlations with their developmental stages.

Melatonin has been detected in several plant families, including Violaceae, Pentaphylacaceae, Juglandaceae, Lamiaceae, Rutaceae, Hypericaceae, Berberidaceae, Piperaceae, Ranunculaceae, and Asteraceae. Within these families, melatonin levels can vary widely, ranging from undetectable to significantly high, with an estimated average concentration of 579.38 ± 1513.28 ng per gram of fresh weight [5]. This variation in melatonin levels supports our selection of Liberica and Robusta coffee as comparative test plants to investigate the potential of melatonin as a growth indicator during the vegetative phase.

2 Materials and methods

2.1 Coffee plant preparation and leaf area observations

Coffee seedlings purchased from farmers undergo acclimatization by providing shade with para-net materials. Periodic monitoring and watering of the plants were conducted to prevent pest infestations, stress conditions, and wilting. Leaf area observations were conducted on five young, middle-aged, and old Robusta and Liberica coffee leaves. Each leaf's most extended and expansive dimensions were measured using a ruler, and the leaf area was calculated using specialized leaf area software for Android.

2.2 Fresh leaf powdering

In the morning, twelve fresh leaves from each part of Robusta and Liberica coffee plants—upper, middle, and lower—were collected. The leaves were rolled and cut into small pieces, then ground with liquid nitrogen using a mortar and pestle. This method is commonly employed to extract intracellular compounds [6].

2.3 Leaf extraction

The obtained coffee powder was mixed with 70% methanol solvent in a ratio of 1:5 [7]. The solution was shaken every two hours for 24 hours as part of the maceration process. After maceration, the mixture was filtered through fine filter paper with 15-20 µm pore sizes. The methanol solvent was then evaporated using a rotary evaporator at a temperature of 45-55°C and a rotation speed of 96 rpm. The resulting thick extract was stored in a tightly sealed 100 ml dark bottle and refrigerated for further analysis.

2.4 Thin layer chromatography analysis

Thin Layer Chromatography (TLC) analysis was conducted by applying 5 µl of coffee leaf extract to a silica gel F_{254} plate using a microcapillary. The silica gel plate was then eluted with a mobile phase consisting of Butanol: Acetic Acid: Water in a ratio of 12:3:5. Synthetic melatonin at a concentration of 2 mg/ml served as a reference standard. After elution, the TLC plate was dried, and the spots were examined under UV light at a wavelength of 254 nm [8].

2.5 Liquid Chromatography-Mass Spectrometry analysis

Liquid chromatography-mass spectrometry (LC-MS) analysis was conducted using the Surveyor LC system, integrated with an Ion Trap LCQ DECA XP Plus mass spectrometer. A sample volume of 5 µL was injected into a Synergi Hydro 4 µm C18 column maintained at 37°C. The mobile phase consisted of a mixture of ammonium formate/formic acid buffer and acetonitrile, which was delivered at a flow rate of 0.3 mL/min using a linear gradient. The scanning process was performed in total ion mode and MS/MS, covering an m/z range of 100-2000. The intensity of the ions detected at each m/z value was recorded to produce a mass spectrum. The resulting mass spectrum was further analyzed using single reaction monitoring (SRM) mode [9]. LC-MS was only performed on the extract of young and middle-aged leaves.

2.6 Data analysis

Research data are presented descriptively, highlighting the results of leaf area measurements and observations of leaf melatonin using Thin Layer Chromatography (TLC) and Liquid Chromatography-Mass Spectrometry (LC-MS).

3 Results and discussions

3.1 Plant leaf area

In our study (Table 1), the leaf areas of young, middle-aged, and old Liberica coffee leaves were measured at 59.29 cm^2, 98.44 cm^2, and 59.01 cm^2, respectively, with the largest area observed in middle-aged leaves. This finding supports the idea that leaves achieve optimal size and photosynthetic capacity during this developmental stage, enhancing light interception and resource allocation, which are crucial for photosynthesis and melatonin synthesis and vital for plant growth and stress responses [4]. Using light energy, photosynthesis transforms carbon dioxide and water into organic compounds and oxygen, a process vital for all living organisms on our planet. In plants, reactive oxygen species (ROS) are by-products of oxygen during photosynthesis and oxidative phosphorylation in chloroplasts, where phytomelatonin is primarily produced. The production of phytomelatonin can be triggered by light and exhibits daily rhythms, peaking during the day, which may be crucial for scavenging excess ROS. In addition to directly shielding chloroplasts from excessive ROS stress, phytomelatonin serves as a signaling molecule that regulates chlorophyll and protein synthesis and degradation, photosynthetic rates, and the metabolism of sugars, lipids, and secondary metabolites by modulating the transcription of related genes and interacting with hormone signals. In contrast to Liberica, Robusta coffee leaves measured 46.32 cm^2, 60.10 cm^2, and 26.03 cm^2 for young, middle-aged, and old leaves, respectively. While middle-aged leaves in Robusta also exhibited the largest area, the overall measurements were consistently lower than those of Liberica. The significant reduction in leaf area in old Robusta leaves may limit photosynthetic capacity and melatonin synthesis, suggesting different growth strategies and environmental adaptations between the two species.

Table 1. Area of *Coffea liberica* and *Coffea canephora* leaves at different leaf ages

Species	Leaf area (cm^2) mean±stdv		
	Young	Middle	Old
Coffea liberica	59.29±23.12	98.44±35.43	59.01±19.71
Coffea canephora	46.32±13.50	60.10±18.95	26.03±15.71

Comparing Liberica and Robusta coffee, it is clear that Liberica exhibits larger leaf areas across all developmental stages, indicating a greater capacity for light interception and photosynthetic efficiency. These differences likely reflect distinct growth environments and adaptive strategies, with Liberica thriving in conditions that favor larger leaf structures.

3.2 Qualitatively presence of melatonin

A positive TLC test for melatonin is indicated by the presence of spots corresponding to the expected Rf (retention factor) value of melatonin compared to a synthetic melatonin standard. These spots should exhibit similar colors or fluorescence under UV light. In our study, all leaf samples from young, middle, and old age of Liberica and Robusta coffee exhibited spots, confirming that melatonin is present in each leaf stage (Table 2).

Table 2. Qualitatively melatonin presence in *Coffea liberica* and *Coffea canephora* leaves at different leaf ages

Species	Melatonin		
	Young	Middle	Old
Coffea liberica	+	+	+
Coffea canephora	+	+	+

+: Detected.

As indicated by the TLC test, the presence of melatonin in all leaf samples from young, middle-aged, and old Liberica and Robusta coffee suggests that melatonin plays a crucial role throughout the various stages of leaf development. Melatonin is known for its involvement in numerous physiological processes in plants, including growth regulation, stress response, and antioxidant activity. Its consistent presence across different leaf ages may reflect its function in promoting healthy growth and protecting against environmental stresses [4, 10]. The identification of melatonin in all leaf stages underscores its importance in the life cycle of coffee plants, suggesting that its presence is vital for optimizing growth and enhancing the plant's ability to adapt to varying environmental conditions.

3.3 Melatonin concentrations in *Coffea liberica* and *Coffea canephora* leaves

Our study's LC-MS results indicated that the melatonin concentrations in young and middle-aged Liberica coffee leaves were 0.72% and 0.58%, respectively. In Robusta coffee, the concentrations were slightly higher in the young leaves at 0.73% and lower in the middle leaves at 0.51% (Table 3). The higher concentration of melatonin in young leaves may indicate its critical role in promoting growth and development, as melatonin is known to enhance photosynthetic efficiency and stress tolerance. Conversely, decreased melatonin concentration in middle-aged leaves could suggest a shift in metabolic functions or a response to environmental conditions.

Table 3. Percentage of melatonin concentration in *Coffea liberica* and *Coffea canephora* leaves at different leaf ages

Species	Melatonin concentration (%)		
	Young	Middle	Old
Coffea liberica	0.72	0.58	n.p.
Coffea canephora	0.73	0.51	n.p.

n.p.: Not performed

As leaves age, their physiological roles change, and they may become more focused on maintenance rather than growth. This decline in melatonin levels could indicate a reduced need for growth-promoting compounds, which in turn might suggest a shift in the plant's energy allocation from growth to other functions. This could be an adaptation to changing environmental conditions, as middle-aged leaves might redirect resources toward other functions, such as nutrient storage or defense against pathogens. This dynamic nature of melatonin's role underscores its multifaceted involvement in plant physiology, adapting to the needs of the plant as it progresses through different developmental stages.

Moreover, the differences in melatonin concentrations between Liberica and Robusta coffee highlight potential species-specific adaptations. The generally higher levels of melatonin in both young and middle-aged leaves of Liberica coffee could suggest an enhanced capacity for photosynthesis and stress resilience compared to Robusta [11]. Understanding these variations can provide insights into different coffee species' ecological and physiological strategies, particularly in response to environmental pressures.

In summary, the varying melatonin concentrations across leaf ages in both Liberica and Robusta coffee underscore the importance of this hormone in plant development and adaptation. Future research should explore melatonin synthesis's regulatory mechanisms and interactions with other plant hormones to further elucidate its role in coffee plant physiology.

4 Conclusions

This research investigates the leaf melatonin profile in Liberica and Robusta coffee plants, exploring its potential as a growth indicator during the vegetative phase. We hypothesized that the melatonin profiles would vary with leaf age and could be effectively detected using Thin Layer Chromatography (TLC) analysis. However, our findings indicated that significant variations in melatonin concentrations between young and middle-aged leaves were only detectable through Liquid Chromatography-Mass Spectrometry (LC-MS) analysis. The data generated from this study suggest that melatonin concentration in leaves can serve as a valuable indicator of vegetative growth. However, further research is necessary to optimize analytical methods for more comprehensive assessments.

The authors sincerely thank the Faculty of Mathematics and Natural Sciences, Universitas Brawijaya, for facilitating this research under Internal Research Funding with contract No. 2612.37/UN10.F09/PN/2024.

References

1. L. Hakim, Agroforestri Kopi: Mendorong Taman Hayati dan Wisata Kopi (Media Nusa Creative, 2021).
2. P. Prasetyo, R. Hidayat, Nyoto, H. Purnomo, Budidaya kopi liberika di lahan gambut. Center for International Forestry Research (CIFOR). **2019**,1–4 (2019).
3. W.B. Sunarharum, D.J. Williams, H.E. Smyth, Complexity of coffee flavor: A compositional and sensory perspective. Food Research International. **62**, 315–325 (2014). https://doi.org/10.1016/j.foodres.2014.02.030.
4. X. Xie, D. Ding, D. Bai, Y. Zhu, W. Sun, Y. Sun, D. Zhang, Melatonin biosynthesis pathways in nature and its production in engineered microorganisms. Synthetic and Systems Biotechnology. **7**, 544–553 (2022). https://doi.org/10.1016/j.synbio.2021.12.011.
5. G. Mannino, C. Pernici, G. Serio, C. Gentile, C.M. Bertea, Melatonin and phytomelatonin: Chemistry, biosynthesis, metabolism, distribution and bioactivity in plants and animals—an overview. International Journal of Molecular Sciences. **22**, (2021). https://doi.org/10.3390/ijms22189996.
6. P. Ebrahimi, D. Mihaylova, C.M. Marangon, L. Grigoletto, A. Lante, Impact of sample pretreatment and extraction methods on the bioactive compounds of sugar beet (*Beta vulgaris* L.) leaves. Molecules. **21**, 8110 (2022). doi: 10.3390/molecules27228110. PMID: 36432211; PMCID: PMC9697780.
7. D.Y. Pristiana, S. Susanti, Nurwantoro, Antioksidan dan kadar fenol berbagai ekstrak daun kopi (*Coffea* sp.): Potensi aplikasi bahan alami untuk fortifikasi pangan. Jurnal Aplikasi Teknologi Pangan.**6**, 89-92 (2017).
8. W. Widyaningsih, N. Salamah, F.Q. Maulida, The effects of ethanolic extract of green algae (*Ulva lactuca* L.) on blood cholesterol levels in male rats induced by a high fat diet. JKKI. **7**, 181-186 (2016).
9. G. Allegrone, F. Razzano, F. Pollastro, G. Grassi, Determination of melatonin content of different varieties of hemp (*Cannabis sativa* L.) by liquid chromatography tandem mass spectrometry. SN Applied Sciences.**1**, 1-8 (2019).
10. M.B. Arnao, J. Hernández-Ruiz, Growth conditions influence the melatonin content of tomato plants. Food Chemistry. **138**, 1212–1214 (2013). https://doi.org/10.1016/j.foodchem.2012.10.077.
11. Y.P. Zhang, S.J. Yang, Y.Y. Chen, Effects of melatonin on photosynthetic performance and antioxidants in melon during cold and recovery. Biologia plantarum. **61**, 571-8 (2017).