Exogenous melatonin applications on acid rain stress mitigation in tea (*Camellia sinensis*)

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ABSTRACT

Rapid industrial development, motor vehicle transportation, volcanic eruptions, fossil fuel use, and increased acid gas emissions exacerbate acid rain, which directly damages plants and inhibits photosynthesis. This damage includes morphological, anatomical, and photosynthetic pigment changes. Melatonin prevents macromolecular damage, plays a role in regulating primary and secondary metabolism, including gene transcription, and enzyme activity, to reduce damage caused by abiotic stress. This study aimed to investigate the internal application interval and concentration of melatonin in tea plants to increase stress tolerance and reduce the impact of acid rain. This study was designed using split-plots, melatonin application intervals of 1, 3, and 5 days, and melatonin concentrations of 0, 50, 100, and 150 µM. Observation data were analyzed using Analysis of Variance (ANOVA). The first factor was tested using the Least Significant Difference (LSD) test, while the second factor and the interaction of both factors were tested using orthogonal polynomials. The results showed that melatonin administered every 3 days significantly delayed the onset of necrosis and reduced damage to leaves and stomata. A concentration of 119.85 µM delayed necrosis the longest, a concentration of 104 µM minimized the percentage of leaf damage, and a concentration of 109.29 µM minimized the percentage of stomatal damage. The interaction between the application interval and melatonin concentration influenced each other in increasing tea plant tolerance to acid rain stress. The 3-day interval and 100 µM concentration maintained the anatomical integrity of tea leaves and were most effective in increasing chlorophyll a, total chlorophyll, chlorophyll ratio, and total carotene levels in tea plants. These findings provide direct guidance for tea farmers and environmental management practitioners to reduce the impact of acid rain stress. By applying melatonin at the recommended interval and concentration, farmers can increase the resistance of tea plants, maintain productivity, and mitigate economic losses due to acid rain stress.

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1. INTRODUCTION

Tea plants (*Camellia sinensis*) are one of Indonesia's leading agricultural commodities, classified as perennial plants and considered refreshment plants. Tea leaves are used to make beverages that have a refreshing effect and a pleasant taste. Decreased plant productivity is influenced by two factors: biotic and abiotic factors. If these factors do not meet the growth requirements, they can cause stress on the plants. Stress refers to deviations in physiology, development, and function that cause damage to plants and the environment.

Abiotic stress increases redox status, leading to higher accumulation of Reactive Oxygen Species (ROS) and Reactive Nitrogen Species (RNS), thereby reducing growth and increasing damage to plant macromolecules [1]. Acid rain can damage the morphology of tea plants, causing necrosis and corrosion spots on the leaf surface. Treatment with acid rain at a pH of 3.5 resulted in a decrease in photosynthetic pigments chlorophyll a, chlorophyll b, and carotenoids by 6.6%, 8.5%, and 10.9%, while treatment at pH 2.5 resulted in decreases of 38.4%, 55.3%, and 57.7% [2]. Acid rain at a pH of 2.5 caused damage to the epidermal tissue, particularly the intercostal region, the edges of the adaxial and abaxial surfaces, damage to the wax layer, deformation of peltate trichomes, loss of the cuticle and chloroplasts at the leaf edges, and significant damage to the palisade parenchyma [3]. Acid rain is an ecological factor that greatly affects the decline in crop productivity caused by air pollution from volcanic eruptions, industrialization, fossil fuels, power plants, and motor vehicles.

Plant performance, which can be analyzed based on morphological, anatomical, and photosynthetic pigment characteristics of leaves, is one of the factors for assessing plant resilience under acid rain stress. Leaves play an important role in the process of photosynthesis, because leaves are the organs where photosynthesis occurs [4]. Stomatal density, quantity, and opening play a role in CO_2 absorption for photosynthesis and water loss through transpiration, which are interrelated processes. Thus, stomata can influence plant biomass production even under fluctuating solar radiation due to their connection with CO_2 supply and water use efficiency in photosynthesis [5]. The more stomata there are, the higher the mechanism for releasing water from cells will be. High turgor pressure triggers guard cells to stretch and open stomatal pores until cell turgor pressure approaches the minimum threshold, thereby preventing the plant from experiencing waterlogging [6]. Stomata are cellular structures on the leaf epidermis that facilitate gas exchange (CO_2 and H_2O) between the plant and the atmosphere/environment.

Chloroplasts are organelles that carry out photosynthesis using energy from sunlight to synthesize ATP and NADPH, compounds that are used to support CO_2 fixation reactions in the stroma, known as the Calvin-Benson cycle, for the formation of carbohydrates. Chloroplasts play a role in photosynthesis in the green parts of plants, which is due to the chlorophyll pigment in the chloroplast membrane. Sunlight has varying energy levels, which are influenced by wavelength. Chlorophyll a absorbs wavelengths from 420 nm to 660 nm, chlorophyll b absorbs wavelengths from 440 nm to 640 nm, while β-carotene absorbs wavelengths from 425 nm to 470 nm. The combination of various pigments makes the spectrum of sunlight energy absorption wide [7]. Photosynthesis is a biochemical process involving the conversion of H_2O and the absorption of CO_2 from the atmosphere to produce complex organic molecules such as carbohydrates and O_2 through the conversion of light energy into chemical energy [8].

Melatonin (N-acetyl-5-methoxytryptamine) is a multifunctional molecule that is universally distributed in various plant organs to strengthen various physiological mechanisms, fight disease, and increase tolerance to ecological stress. Melatonin biosynthesis occurs in plants under stress conditions, with concentrations varying from plant to plant and even varying between different plant organs [9]. Exogenously applied melatonin increases tolerance to several environmental stresses through changes in gene expression in various abiotic stress response pathways, resulting in increased tolerance, including acid rain, low temperature, drought, heavy metal toxicity, and salinity. Melatonin is a biodegradable pleiotropic molecule that is non-toxic to humans. Exogenous melatonin application induces changes in gene expression in various abiotic stress response pathways, resulting in increased tolerance, including acid rain stress, low temperature, drought, heavy metal toxicity, and salinity [10]. Melatonin induction in plants experiencing acid rain stress showed that leaf mesophyll cells had regular chloroplast shapes, well-organized grana starch in chloroplasts, and no cell wall damage. Melatonin treatment showed the highest recovery of chlorophyll a, chlorophyll b, and chlorophyll fluorescence, namely 20.46%, 23.08%, and 2.44% [11].

Melatonin has emerged as a promising solution and recent development in mitigating environmental stress in plants. This multifunctional molecule naturally strengthens physiological mechanisms and increases tolerance to various ecological stresses. Exogenous melatonin application has been shown to trigger gene expression changes in abiotic stress response pathways, maintain leaf mesophyll cell integrity, and even restore chlorophyll levels. However, the novelty of this study lies in its contribution to identifying the most optimal internal application and concentration of melatonin to improve the performance and productivity of tea plants as a mitigation of acid rain stress to maximize the effectiveness of melatonin.

2. METHODS

The research was conducted at PT Perkebunan Tambi, and anatomical parameter observations were carried out at the Agronomy and Physiology Laboratory of the UPA Agrotechnology Park at Tidar University from February 17 to April 11, 2025. The tools used for analysis were a pH meter, spectrophotometer, leaf area meter, binocular microscope, optilab, and image raster. The materials used were 1-year-old grade A Gambung

7 tea seedlings, melatonin, 98 % H2SO4, 98 % HNO3, 3.5 pH buffer distilled water, and 80% ethanol. The research was conducted using a factorial experiment (3×4) arranged in a split-plot design. There were two treatment factors, namely the first factor of melatonin application interval as the main plot, and the second factor of melatonin concentration as the subplot. The research was repeated three times. The primary data of the experiment were analyzed using Analysis of Variance (ANOVA) with a confidence level of 5% and 1%. Significant and very significant results were subjected to further testing. The first factor, the interval of melatonin application, was subjected to a Least Significant Difference (LSD) test, while the second factor, melatonin concentration, and the interaction between the first and second factors were subjected to an orthogonal polynomials test. The experimental procedure is shown in Fig. 1.

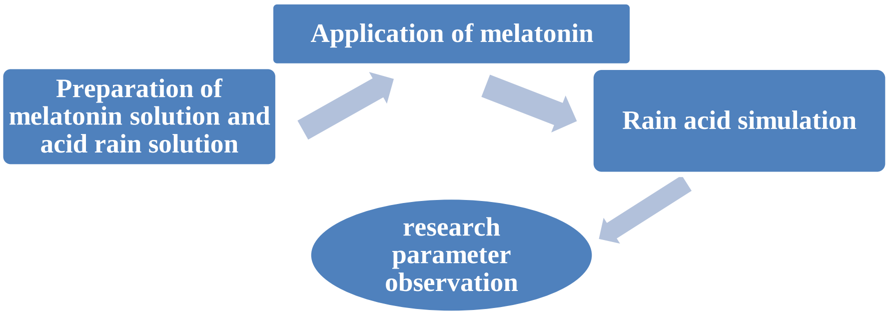


Fig. 1. Flowchart of the experimental procedure

2.1. Preparation of melatonin solution

Melatonin is produced by opening melatonin capsules using a cutter, then dissolving them in distilled water. The µM concentration is produced by converting to ppm using the following formula (1).

$$Ppm = \frac{\mu M\ X\ Molecular\ weight\ of\ melatonin}{1000} \quad (1)$$

2.2. Application of melatonin

The melatonin solution was sprayed evenly on the leaves using a hand sprayer at a dose of 20 ml/plant and watered with 30 ml/polybag, covered with a box chamber to prevent contamination in other combinations [12]. The application was carried out in the morning to prevent rapid evaporation and maximize the absorption of exogenous melatonin. The application of exogenous melatonin requires low light conditions and moderate temperatures below 28 ℃ to increase absorption and prevent melatonin degradation. These intervals were chosen to explore the duration of melatonin's effectiveness in plant tissue. The 1-day interval represents frequent application to maintain consistently high melatonin levels. The 3-day interval is considered a midpoint, balancing the need for sustainability of effects with melatonin efficiency. Meanwhile, the 5-day interval was tested to determine whether the effects of melatonin are still significant over a longer period, which may reduce the frequency of application in the field. This selection aims to identify the optimal balance between effectiveness and efficiency of application. The selection of intervals and concentrations was systematically designed to identify the most effective and efficient concentrations and application intervals for improving tea plant tolerance to acid rain stress, providing robust data for practical recommendations.

2.3. Preparation of acid rain solution

The acid rain solution was prepared from 98% H2SO4 and 98% HNO3 diluted with distilled water. The dilution method was carried out by determining the molarity of H2SO4 and HNO3 required and the volume of distilled water used for dilution to obtain an acid solution with a pH of 3.5 by adding a pH 3.5 buffer solution to stabilize the pH.

2.4. Rain acid simulation

Tea plants were sprayed with acid rain solution at 20 ml/plant [13]. 11 applications over 16 days based on the average of rainy days in Wonosobo Regency in 2022, which showed 20 rainy days/month in a year.

2.5. Parameters necrotic appearance time

The observation of the initial appearance of necrosis was carried out by counting the days when necrosis began to appear, characterized by brown or black spots on the upper and lower surfaces of the leaves, indicating tissue death. The appearance of necrosis was observed daily on the upper and lower surfaces of the leaves, starting after the acid rain simulation.

2.6. Percentage of leaf damage (%)

The percentage of leaf damage was measured using a leaf area meter on the upper and lower epidermis. The leaves tested were leaves from sample plants located on the second layer of leaves from the top growing.

2.7. Stomatal density (unit/mm^2)

Stomatal density was observed on the lower epidermis of the third leaf layer from the upper growing point using the stomatal printing method. Stomatal density was observed using a binocular microscope and counting the number of stomata in the field of view at 40x magnification.

2.8. Percentage of stomatal damage (%/mm^2)

Stomatal damage was observed on the lower epidermis of the third leaf layer from the upper growing point using the stomatal printing method. The percentage of stomatal damage was observed using a 40x magnification binocular microscope and calculating the percentage of damaged stomata.

2.9. Anatomical appearance of the upper surface of leaves

Observation of the anatomical appearance of the upper surface was carried out on the third leaf layer from the upper growing point, microscopically using the printing method with a 40x magnification binocular microscope, observing the symptoms of damage to the upper leaf surface layer 4 days after harvest.

2.10. Anatomical appearance of cross-sectional leaf structure

The cross-sectional structure of the leaf was observed by making a horizontal cut on the third layer of the leaf from the upper growth point at the border between the fresh leaf and the necrotic leaf to obtain a cross-sectional preparation of the tea leaf. The observation was carried out by placing the leaf preparation on an object glass and covering it with a cover glass. Observations were made using a binocular microscope at 100x magnification, examining the cuticle, upper epidermis, palisade parenchyma, vascular bundles, spongy parenchyma, and lower epidermis.

2.11. Photosynthetic pigments

The photosynthetic pigment content was tested by measuring chlorophyll a, chlorophyll b, total chlorophyll a + b, chlorophyll a/b ratio, and total carotene. Photosynthetic pigment testing uses the ninhydrin principle based on a spectrophotometer. The method used to determine chlorophyll and carotenoids is as follows: 50 mg of fresh tea leaves are cut into several filaments with a length of ≥20 mm and a width of ≤1 mm along the central vein. The leaf samples were dissolved in 10 ml of 80 % ethanol and incubated for 12 hours. The extract was filtered and analyzed using a spectrophotometer, and the absorbance was recorded at wavelengths of 645 nm for chlorophyll a, 663 nm for chlorophyll b, and 470 nm for total carotenoids [14].

3. RESULTS AND DISCUSSION

Based on the data analysis, the F value calculated for all observation parameters presented in Table 1. The F-value in Table 1 shows that the intervals application of melatonin does not affect leaf stomatal density, but acid rain stress changes the stomatal arrangement to become irregular, even though the number of stomata per unit leaf area remains the same. This shows that acid rain can interfere with the stomatal development process and cause structural changes in the leaves, even though it does not affect the overall number of stomata. Tea plants propagated vegetatively will have similar phenotypic characteristics, expressed from the genes they possess, so plants with the same genetic makeup will exhibit similar phenotypes, including leaf stomatal density. Stomatal density is more influenced by plant age and leaf position number. Genetic and environmental factors affecting stomatal formation act uniformly at the same age and leaf position, resulting in consistent stomatal density.

Table 1. Calculated F values for all observation parameters

Research Parameters	F Value		
	P	K	P X K
Necrotic appearance time	392,91**	558,73**	25,89**
Percentage of leaf damage (%)	41,15**	165,14**	4,84**
Stomatal density (unit/mm^2)	1,53^{ns}	0,55^{ns}	0,73^{ns}
Percentage of stomatal damage (%/mm^2)	7,98*	96,32**	4,63**

Application of melatonin to apple plants under conditions of stomatal stress keeps the stomata open, but the density of stomata on the leaves is not changed by melatonin. Abiotic stress on apple plants pretreated with melatonin resulted in longer and wider stomata than the control plants, which showed much shorter, narrower stomata and caused stomatal closure. Stomatal density is thought to be influenced by plant clone genetics, so that the same clone will show uniformly expressed phenotypes due to precise genetic transcription, so that the propagation results will be identical to the parent unless a mutation occurs. The application of exogenous melatonin 3 times at all application intervals did not change the number of stomata, so that the density remained unchanged [15].

State that vegetative propagation involves theoretical duplication, where the selected genotype maintains its original genetic composition through mitotic division, ensuring that all individual characteristics are well-inherited. This indicates that the genes encoding stomatal number are maintained as a clonal trait, so that identical clones exhibit uniform stomatal density. Stomata are microscopic pores on the plant epidermis that function as the primary pathway for gas and water exchange between plants and the atmosphere [16]. Stomatal characteristics, including size, conductance, and density, result from the interaction between stomatal morphology and plant physiological activity influenced by the environment to stabilize cell turgor and prevent water excess or deficiency within cells [17]. Environmental factors influence plant growth and development conditions, intervals factors originate from plant genetics and trait inheritance, while external factors originate from the environment.

Stomatal density is influenced by genetics, which determine stomatal size and density, and interactions with environmental factors. Environmental conditions such as humidity, temperature, and light significantly affect stomatal activity, plants can adapt to seasonal changes through modifications in stomatal behavior. Tea plants planted at the same time will undergo similar growth and development processes, resulting in relatively similar phenotypic levels. It is hypothesized that factors influencing stomatal density, such as growth hormones, light, and nutrients, will act similarly on all plants.

Different degrees of acidity (pH) from sulfuric acid affect the toxicity it causes; the lower the pH value, the greater the damage caused by sulfuric acid and nitric acid solutions on plants [18]. Tea requires acidic pH for optimal growth; neutral pH causes brown roots and slow growth compared to roots of plants grown with aluminum to lower pH. At a pH of 4.0, tea roots grow stronger and are mostly white [19].

The results of the study indicate that exposure to acid rain with a pH of 3.5 does not reduce the number of stomata but only disrupts their arrangement, making them irregular. Acid rain can disrupt the development process of stomata and cause structural changes in leaves, although it does not affect the total number of stomata. Acid rain with a pH of 3.5 does not reduce the density of tea leaf stomata but causes structural damage to the stomata. Tea plants have growth requirements at a pH of 4.5–5.6 and are believed to have special adaptive mechanisms for acidic environmental conditions. Tea plants grow optimally in Andisol, Podsolik, and Latosol soils. The soil has a crumbly, loose structure, high organic matter content, a pH of 4.5–5.6, and no limestone.

3.1 Intervals application of melatonin to increase tea plant tolerance to acid rain stress

The intervals application of melatonin had a significant effect on the onset time of necrosis, the percentage of leaf damage, and a significant effect on the percentage of stomatal damage. The results of the LSD test on the intervals application of melatonin on the onset time of necrosis and the percentage of stomatal damage can be seen in Table 2.

Table 2. The effect of intervals melatonin application on necrosis, leaf damage area, and stomatal damage

Intervals of application melatonin	Necrotic appearance time	Percentage of leaf damage (%)	Percentage of stomatal damage (%/mm^2)
once a day (P_1)	4,25^c	57,03^a	47,31^a
every three days (P_2)	8,50^a	36,74^b	39,46^b
every five days (P_3)	7,58^b	47,20^a	39,50^b
LSD value	0,85 (α 1 %)	11,89 (α 1 %)	7,25 (α 5 %)

Note: Numbers followed by the same letter in the same column are not significantly different at the 5% and 1% LSD levels.

Based on the results of the LSD test in Table 2, it shows that the intervals application of melatonin every 3 days with a longer average time of necrosis, namely 8.50 days after acid rain simulation, the percentage of leaf damage area is smaller, namely 36.74%, and the average percentage of stomatal damage is 39.46%, but the percentage of stomatal damage was not different from the 5-day interval. After conducting a correlation test between the initial time of necrosis and the percentage of leaf damage, a negative correlation coefficient of 0.94 was obtained. The correlation test between the initial time of necrosis and the percentage of stomatal damage obtained a negative correlation coefficient of 0.97. This indicates an inverse or opposite relationship between the onset of necrosis and the percentage of leaf damage. The sooner necrosis occurs, the higher the percentage of leaf damage and stomatal damage. The correlation test between the percentage of leaf damage area and the percentage of stomatal damage yielded a positive correlation coefficient of 0.94. This indicates a very strong positive relationship between the percentage of leaf damage area and the percentage of stomatal damage. The higher the percentage of leaf damage area, the higher the percentage of stomatal damage.

Necrosis in tea leaves indicates cellular tissue death and morphological damage to the leaves, causing changes in color, texture, and shape, resulting in brownish-black, dry, and brittle leaves due to acid rain stress. The application of melatonin at 3-day intervals significantly reduced leaf damage due to acid rain stress, reaching the lowest damage percentage of 36.74%, while the highest percentage of leaf damage occurred in the 1-day interval treatment, which was 57.03%. This shows that the 3-day intervals interval maintains the optimal melatonin concentration continuously, enabling plants to fight oxidative stress due to acid rain stress.

Acid rain stress at pH 3.5 and pH 2.5 causes visible corrosion and necrotic spots on the leaf surface and inhibits plant growth both above and below ground. The effects of acid rain on plant growth can lead to the formation of characteristic necrotic spots, resulting in phytotoxicity when pH levels reach a certain damage threshold [13]. Increased concentrations of SO_x and NO_x in the atmosphere indicate that pollutants trigger a decrease in rainwater pH, making it more acidic. The resulting acid rain can affect plant cellular pH through the absorption of acidic solutions by stomata. SO_x and NO_x dissolved in plant tissues become nitrites and nitrates and release H+ protons. Stomatal sensitivity to air pollutants increases with decreasing pH [20]. Plants not exposed to acid rain have healthy stomatal cells and epidermal cells. After the acid rain experiment period, microstructural damage was only detectable in the first set of leaves. Epidermal cells of the leaves showed wilting aspects, causing guard cells to undergo structural changes, low stomatal turgor pressure, and epidermal stomatal cells to shrink [21].

The percentage of stomatal damage is closely related to plant physiology, as stomata play a crucial role in vital processes such as photosynthesis, respiration, and transpiration. Stomata are an important part of plants because they are cellular openings on the leaf epidermis surface that act as portals for gas exchange processes involving CO_2 and water between plants and the atmosphere. Stomata influence plant metabolism, including respiration, distribution of elements through leaf suction, and transpiration.

Stomatal damage can have a negative impact on plant growth and productivity, reducing the rate of photosynthesis because CO_2 cannot enter efficiently, disrupting respiration and ATP formation, and causing disturbances in the water balance of plant cells [22]. Acid rain has been shown to have a significant impact on plant physiology through corrosion of the epidermal structure and disruption of chloroplast function. Erosion of the cuticle layer directly disrupts gas exchange regulation and has the potential to affect cuticle layer biosynthesis. The infiltration of hydrogen ions into plant tissues can disrupt chloroplast activity, leading to impaired carbohydrate metabolism characterized by inefficient starch accumulation. Increased uptake of heavy metal ions can trigger metal toxicity within plant cells, disrupting energy control and cellular homeostasis. The photosynthesis process also experiences a decline, resulting in reduced light utilization. Exposure to acid rain triggers a series of complex structural and metabolic dysfunctions, thereby inhibiting plant growth, development, and productivity [23].

External acid rain exposure is detected by receptors on plant cell membranes, triggering complex intracellular responses. One of the effects of acid rain stress is cellular damage and increased ROS, which affect the production of secondary metabolites and the physiological response of plants. Exogenous melatonin application interacts with specific receptors on the cell membrane, which in turn activates antioxidant defense mechanisms. Melatonin plays a role in directly suppressing the increase in ROS, neutralizing free radicals, and reducing the level of oxidative damage. Melatonin can influence signaling pathways involving transcription factors and gene expression, which ultimately contribute to increased antioxidant production, enabling melatonin to mediate adaptive responses at the molecular and physiological levels to overcome the negative effects of acid rain stress [24].

Melatonin is able to regulate the expression of genes involved in toxicity responses, thereby increasing the ability of plants to adapt to acid rain stress conditions through protein structure stability and increased antioxidant gene activity/expression Intervals application of melatonin increases plant resistance to acid rain stress by protecting cells from oxidative damage. Regular application of melatonin at appropriate intervals will ensure the availability of melatonin in target tissues, thereby improving plant performance under acid rain stress [25].

Melatonin is a bioactive compound that has the potential to increase plant resistance to stress and has biodegradable characteristics that can decompose naturally in the environment [26]. Melatonin can freely pass through cell membranes because it has amphiphilic properties, is able to protect cells from damage, modulate stress-responsive transcription factors, and biosynthesize secondary metabolite-related genes to increase plant tolerance to acid rain stress [27].

The right application interval is a key factor in maximizing its effectiveness and minimizing the potential degradation of melatonin before it reaches the target plant tissue. Too long an application interval is thought to cause significant degradation of melatonin, reducing its effectiveness, while too short an interval can cause melatonin to accumulate in the plant, reducing its amphiphilic ability. Intervals application of melatonin increases plant resistance to acid rain stress by protecting cells from oxidative damage. Regular application of melatonin at appropriate intervals will ensure the availability of melatonin in target tissues, thereby improving plant performance under acid rain stress.

3.2 Melatonin concentration for increasing tea plant tolerance to acid rain stress

A. Necrotic appearance time

The results of the orthogonal polynomial test of melatonin concentration treatment on the time of onset of necrosis are presented in Fig. 2. The results of the orthogonal polynomial test of melatonin concentration showed a quadratic equation of y = -0.0007x2 + 0.1678x + 0.2222 and $R^2 = 0.8405$, indicating that melatonin concentration can affect the onset time of necrosis by 84.05%. Based on the quadratic equation, the optimal melatonin concentration was obtained at 119.85 µM with the onset of necrosis occurring on the 10th day after acid rain stress, while the treatment without melatonin showed the onset of necrosis on the 1st day after acid rain. The results showed that necrosis, as a marker of cell and tissue damage in plants due to acid rain, can be prevented by the application of melatonin, which showed significant potential in increasing plant resistance to stress, particularly in this study as a response to acid rain. acid rain simulation generally causes ROS accumulation and weakens the free radical scavenging potential in plants. Excessive ROS accumulation in plant parts is the result of protective mechanisms in tissues stressed by acid rain.

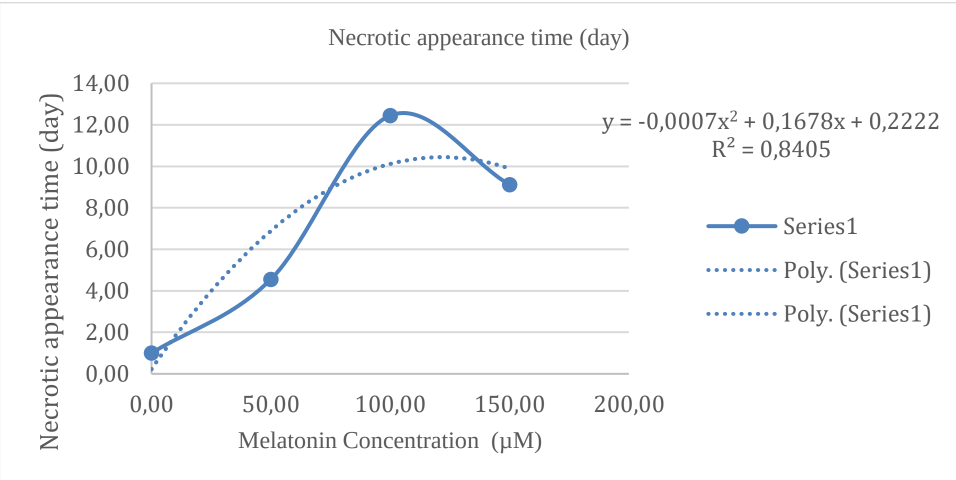


Fig. 2. Melatonin concentration versus time of initial necrotic appearance

Acid rain simulation at pH 4 showed increased antioxidant enzyme activity over ten days of acid rain simulation, but acid rain simulation at pH 3 and pH 2 for one day indicated oxidative stress induction in plants. Chronic exposure to high acidity significantly inhibited the activity of catalase (CAT) and ascorbate peroxidase (APx) enzymes, which positively correlated with the accumulation of malondialdehyde (MDA) as an indicator of lipid peroxidation. The research results indicate that the capacity of the endogenous antioxidant system is insufficient to mitigate cellular damage caused by prolonged acid rain stress, as evidenced by lipid membrane damage, leading to necrosis in the leaves [28]. Melatonin is able to reduce the toxicity of various abiotic stresses on plants, involved in counteracting various stresses through the regulation of antioxidant defense mechanisms and protecting photosynthesis and plant organs. Melatonin suppresses the increase in ROS produced in plants, increases antioxidant enzyme activity, and regulates hydrogen peroxide deposition in plant cells, thereby reducing oxidative damage and preventing necrosis due to plant cell and tissue death [29].

The results showed that a melatonin concentration of 50 µM was proven to be ineffective, presumably because the concentration was too low to trigger a significant biological response in plant cells and tissues. The highest concentration of 150 µM was thought to inhibit the penetration of melatonin into leaf cells. This indicates that there is an optimal threshold; melatonin concentrations that are too low are not effective enough, while concentrations that are too high can interfere with the absorption process and cause negative effects on leaf cells. Melatonin increases plant resistance to acid rain stress by reducing toxicity and slowing tissue necrosis, as evidenced by the delay in the appearance of necrotic symptoms.

B. Percentage of leaf damage (%)

The results of the orthogonal polynomial test of melatonin concentration on the percentage of leaf damage are presented in Fig. 3. The results of the orthogonal polynomial test of melatonin concentration showed a quadratic equation of y = 0.0052x2 - 1.0816x + 82.396 and R^2 = 0.9987, indicating that melatonin concentration can affect the percentage of leaf damage caused by acid rain by 99.87%. Based on the quadratic equation, the optimal melatonin concentration is 104 µM with a leaf damage percentage of 26.15%, while the highest leaf damage percentage was shown in the treatment without melatonin with a leaf damage percentage of 82.73%. This shows that melatonin at a concentration of 104 µM optimally protects plants from damage caused by acid rain and is able to neutralize free radicals, thereby minimizing damage to leaf cells and tissues.

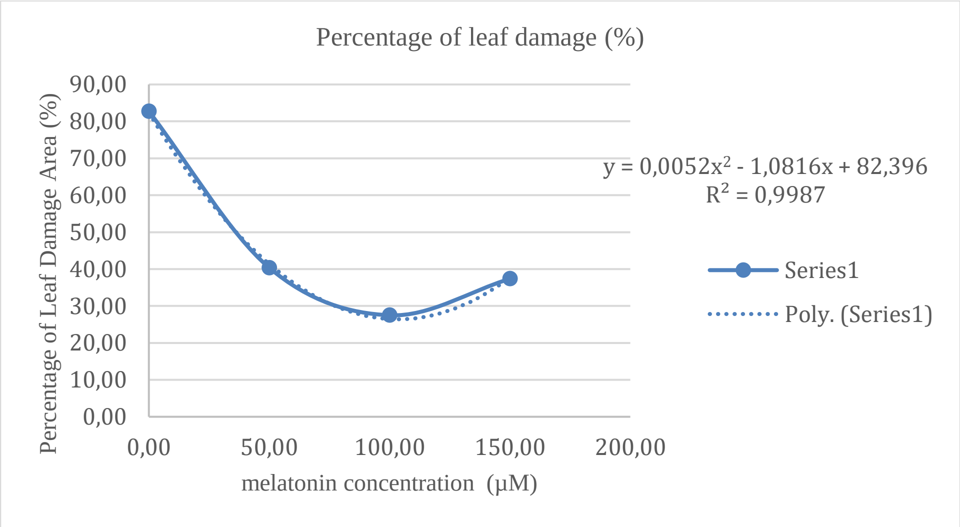


Fig. 3. Percentage of leaf damage area

Melatonin can be synthesized and absorbed by plants to increase resistance to stress. Exogenously application melatonin helps increase tolerance to stress, can bind toxic metals to relieve toxicity, and directly suppresses the increase in ROS and increases antioxidant and enzyme activity to suppress the increase in ROS [30]. Melatonin can increase photosynthetic efficiency, delay leaf senescence, and increase antioxidant enzyme activity, thereby eliminating ROS to increase stress tolerance in *Coffea arabica* plants [31].

Exogenous melatonin regulates enzyme activity and inhibits cadmium (Cd) absorption, and is a new strategy to improve the resistance of tea seedlings or horticultural plants exposed to Cd by applying melatonin as a modulator to weaken the toxicity caused by Cd [32].The optimal concentration of melatonin during two harvest seasons can increase leaf area by increasing cell number and cell size, while the highest concentration suppresses leaf growth and shows an inhibitory effect on chlorophyll synthesis [33].

Exogenous melatonin strengthens the antioxidant defense system and increases soluble protein content, which reduces MDA accumulation and increases tea plant biomass under abiotic stress. The right concentration of melatonin is important in adjusting antioxidant enzyme activity and soluble protein levels to reduce toxicity.

Melatonin reduces Cd accumulation in tea seedlings, thereby retaining large amounts of Cd in the soil [32]. Melatonin induces the biosynthesis of flavonoids and anthocyanins, acting as a regulator and protector against various stresses by activating stress tolerance responses through changes in gene expression. Its anti-stress role is beneficial in agriculture [34]. Stress affects a 55%, 60%, and 45% decrease in plant biomass in the dry weight of roots, stems, and leaves, while plants that were pretreated with 100 µM melatonin before the application of salinity and heat stress showed stable biomass production [35].

C. Percentage of stomatal damage (%/mm2)

The results of the orthogonal polynomial test of melatonin concentration treatment on the percentage of stomatal damage are presented in Fig. 4. The results of the orthogonal polynomial test of melatonin concentration showed a quadratic equation of y = 0.0029x2 – 0.6339x + 63.882 and R^2 = 0.9754, indicating that melatonin concentration can affect the percentage of stomatal damage caused by acid rain by 97.54%.

Based on the quadratic equation, the optimal melatonin concentration was 109.29 µM with a stomatal damage percentage of 29.24%, while the treatment without melatonin showed stomatal damage of 62.96% after acid rain simulation. Higher levels of stomatal damage are likely to have more negative effects on plant physiological activities. The simulated acid rain at pH 3.5 can damage the stomata and stomatal arrangement of tea plants, potentially inhibiting plant growth. Sulfuric acid and nitric acid in acid rain can disrupt stomatal function. Irregular stomatal damage and structure may hinder photosynthesis, respiration, and water balance in plants, thereby reducing tea plant productivity.

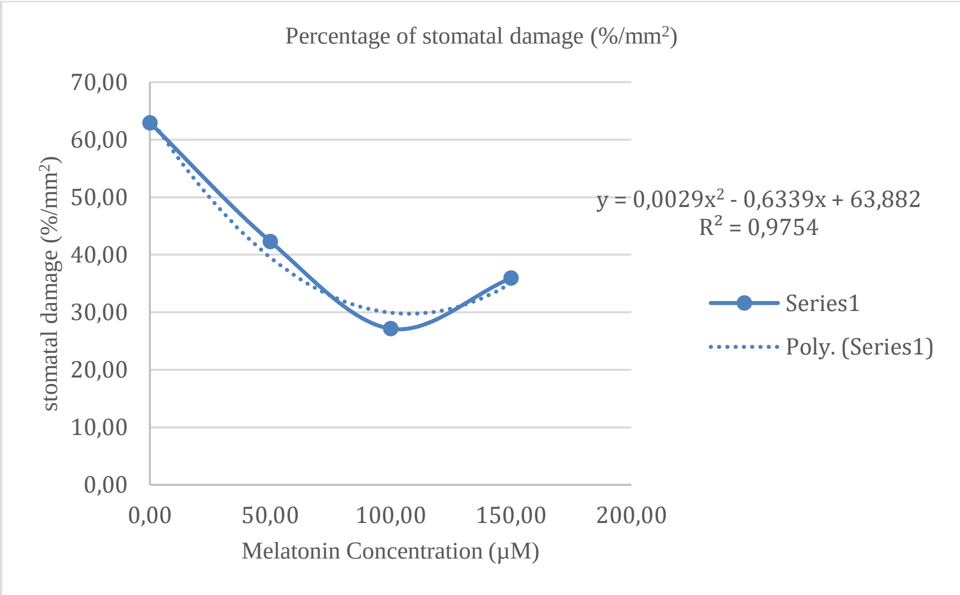


Fig. 4. Melatonin concentration on stomatal damage percentage

Growth inhibition is an undesirable symptom in plants exposed to various abiotic stresses, acid rain stress is suspected to inhibit plant growth due to stomatal disruption, which can result in reduced CO_2 assimilation, disrupted transpiration and respiration, and indirectly affect nutrient and water mobilization within the plant due to impaired leaf suction capacity, thereby disrupting photosynthesis rates and reducing plant productivity. Stomata play a crucial role in the photosynthesis process; when leaf stomata close or their function is disrupted due to damage, it can interfere with the rate of photosynthesis. Stomata significantly influence the transpiration process, as they serve as pathways for water vapor diffusion from leaves to the environment. Stomata open when the turgor pressure of the guard cells is high and close when the turgor pressure of the guard cells is low. Stomata play a role in respiration through the process of releasing or transferring energy from the chemical bonds of organic molecules in living cells to the chemical bonds of ATP. The respiration process requires O2, which occurs in the mitochondria.

Melatonin is able to mediate acid rain stress, and its effectiveness depends on the concentration applied. The results of the study showed that 100 µM exogenous melatonin had the highest efficiency, and the SOD, POD, CAT, and APx values increased by 33%, 30%, 31%, and 19%, respectively, when compared to plants exposed to acid rain without melatonin treatment. Melatonin 100 µM increased acid rain stress tolerance by increasing photosynthesis and ROS-counteracting antioxidant activity in plants compared to other concentration levels. This shows that exogenous melatonin promotes oxidative stress mitigation, which is associated with increased antioxidant activity in tomato plants. the severity of growth inhibition caused by acid rain in tomato seedlings is reduced when melatonin is applied to plants experiencing acid rain stress. Stressed plants treated with 100 µM melatonin recovered faster than plants that were not treated with melatonin [36]. The application of melatonin at a concentration of 100 µM showed a positive response in reducing acid rain toxicity, thereby protecting the stomata from toxic effects. In addition, melatonin with antioxidant activity is thought to be able to improve plant performance under acid rain stress.

3.3 Interaction between application intervals and melatonin concentration for increasing tea plant tolerance to acid rain stress

The interaction between melatonin application and concentration had a significant effect on the onset time of necrosis, the percentage of leaf damage, and the percentage of stomatal damage. The appropriate intervals application of melatonin allowed plants to absorb and distribute melatonin more effectively throughout the plant tissue, resulting in a cumulative protective effect. The optimum melatonin concentration ensures that enough melatonin accumulates to provide optimal defense mechanisms and antioxidant systems. This shows that the two treatment factors influence each other. The right combination of melatonin intervals and concentration can increase the effectiveness and mechanism of action in improving the performance of tea plants under acid rain stress.

A. Necrotic appearance time

The results of the orthogonal polynomial interaction test of the intervals application and melatonin concentration on the time of necrotic appearance are presented in Fig. 5. Figure 4 shows the interaction between the application interval and melatonin concentration on the onset time of necrosis in the acid rain stress simulation. The curve indicates that at low melatonin concentrations, the onset time of necrosis is relatively short. As melatonin concentration increases, the onset time of necrosis tends to increase, reaching a peak before decreasing again. Application with the same concentration but different application intervals showed different effectiveness, indicating that melatonin has the potential to inhibit the onset of necrosis, but its effectiveness is highly dependent on the concentration and application interval.

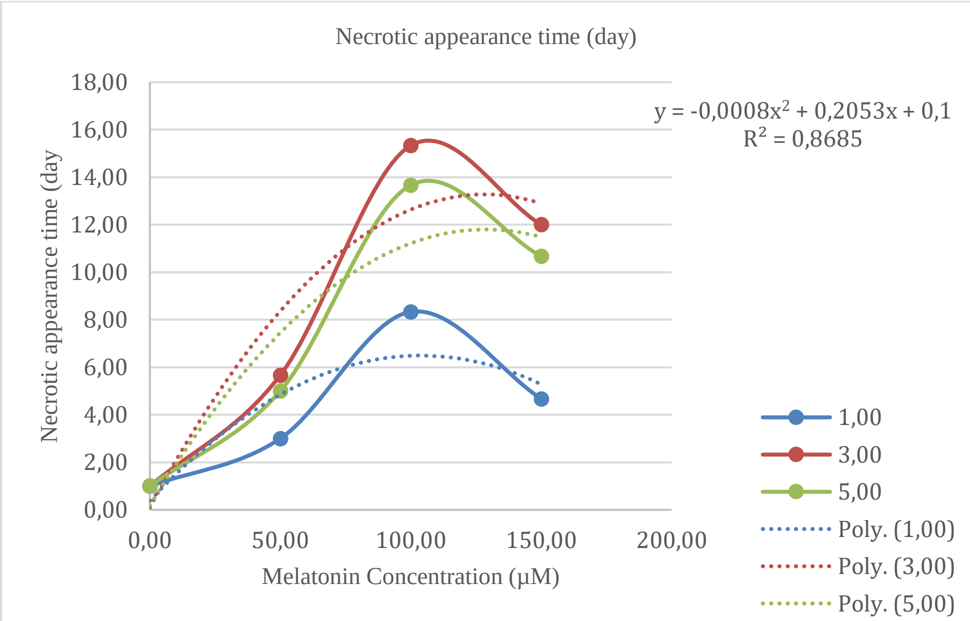


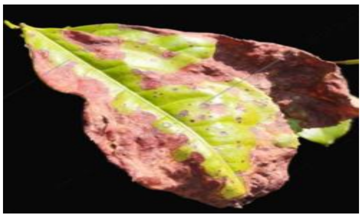
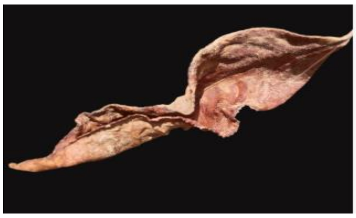
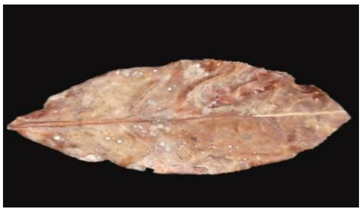
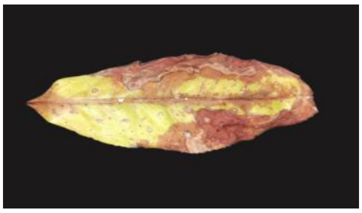
Fig. 5. Interaction between intervals application and melatonin concentration on the time of initial necrotic appearance

The results of the orthogonal polynomial test of melatonin concentration showed a quadratic equation of y = -0.0008x2 + 0.2053x + 0.1 and R^2 = 0.8685, indicating that the interaction between the application interval and melatonin concentration can affect the onset time of necrosis due to acid rain by 86.85%. Based on the quadratic equation, the optimal melatonin concentration was obtained at 128.31 µM with the onset of necrosis occurring on the 13th day after acid rain stress. This shows that the right application interval and melatonin concentration can increase plant resistance to acid rain stress.

Photosynthetic activity, plant growth parameters, and cell ultrastructure damaged by simulated acid rain treatment were significantly improved at an interaction interval of once every 3 days with 100 µM melatonin. The dotted spots seen on tomato leaves due to simulated acid rain treatment were restored by melatonin. The application of melatonin to plants simulated with acid rain stress caused an increase in the regulation of stress-responsive transcription factor genes to increase tolerance to acid rain stress in tomato seedlings [27].

B. Percentage of leaf damage (%)

The interaction between the intervals application and concentration of melatonin affected leaf damage after exposure to acid rain, as shown in Fig. 6.

simulation of acid rain exposure 7 times	simulation of acid rain exposure 11 times
	
P_1K_0	P_1K_0
	
P_2K_0	P_2K_0
	
P_3K_0	P_3K_0
	
P_1K_1	P_1K_1
	
P_2K_1	P_2K_1
	
P_3K_1	P_3K_1

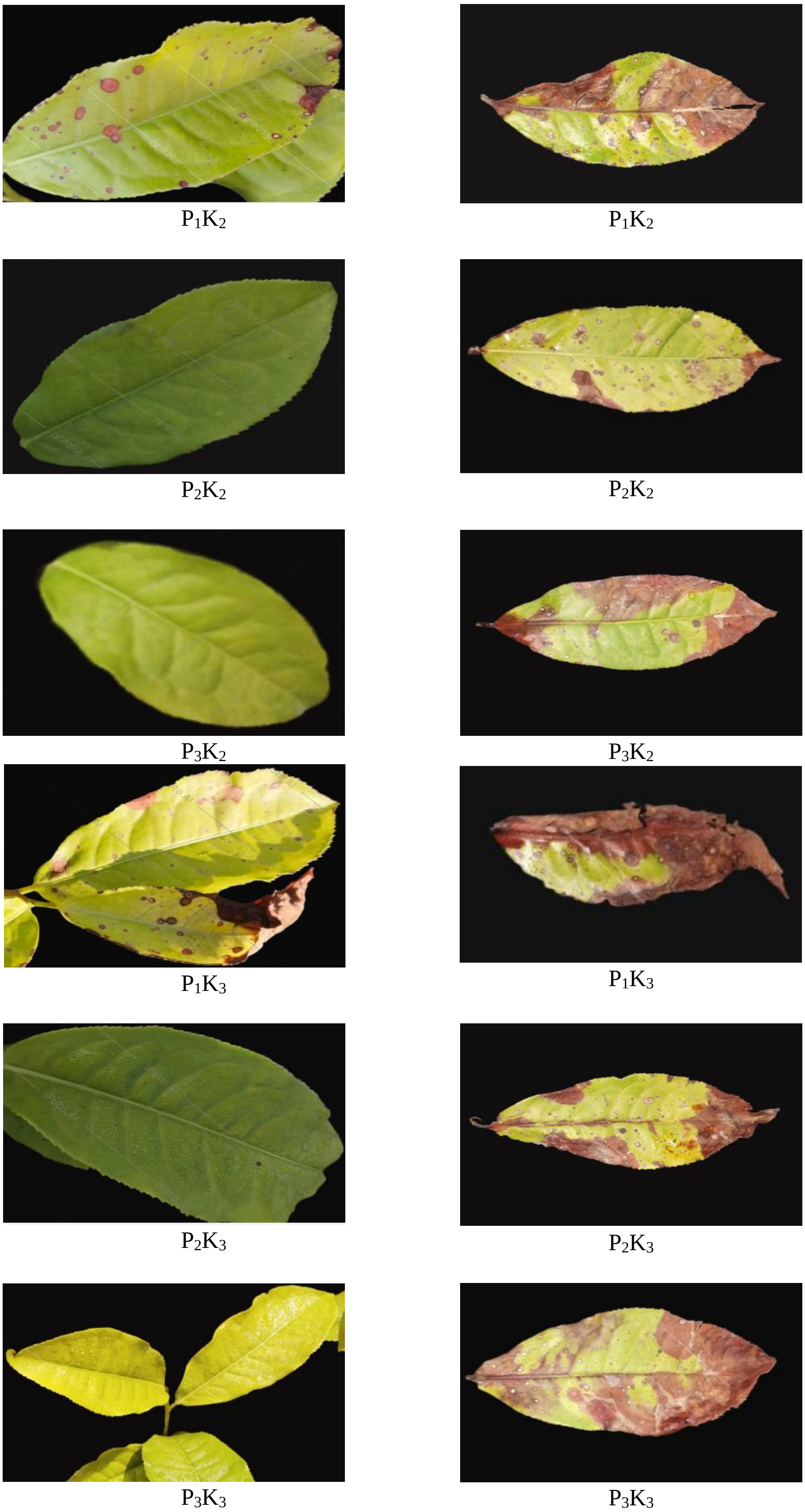


Fig. 6. Damage to tea leaves after 7 and 11 exposures to acid rain

The results of the observations in Figure 5 show that exposure to acid rain seven times significantly caused necrotic damage to the leaves, but there were differences in response between treatments. Treatments P2K2, P3K2, P2K3, and P3K3 did not show signs of necrosis due to acid rain stress, but the leaves turned yellow. This indicates that acid rain can degrade chlorophyll. In line with the results of photosynthetic pigment testing, there was a decrease in chlorophyll a, chlorophyll b, total chlorophyll, and chlorophyll ratio of 65.69%, 50.66%, 58.69%, and 41.91%, respectively, when comparing the highest and lowest treatment values under acid rain stress. Further observations of acid rain exposure over 11 times indicated that all treatments experienced leaf damage with necrotic symptoms, indicating cell and tissue death and potentially disrupting physiological functions due to acid rain stress. The severity of damage varied between treatments, specifically the control treatment showed the most severe damage, indicating the susceptibility of plants without melatonin application to the effects of acid rain stress, while the P2K2 treatment showed the lowest damage.

The results showed that the appropriate application interval and concentration were able to reduce leaf damage caused by acid rain stress, and that more frequent exposure to acid rain affected the level of leaf damage, as well as the potential resistance of certain treatments in the early stages of exposure. Leaf damage tended to increase with increased exposure to acid rain. The damage observed in 7 acid rain events showed a significant increase when it reached 11 acid rain events, indicating that the negative effects of acid rain on leaf integrity worsened over time. The interaction between the intervals and melatonin concentration can mitigate or prevent damage to tea plants, which is very important in plant protection strategies. The results of the orthogonal polynomial test of the interaction between the intervals application and melatonin concentration on the percentage of leaf damage are presented in Fig. 7.

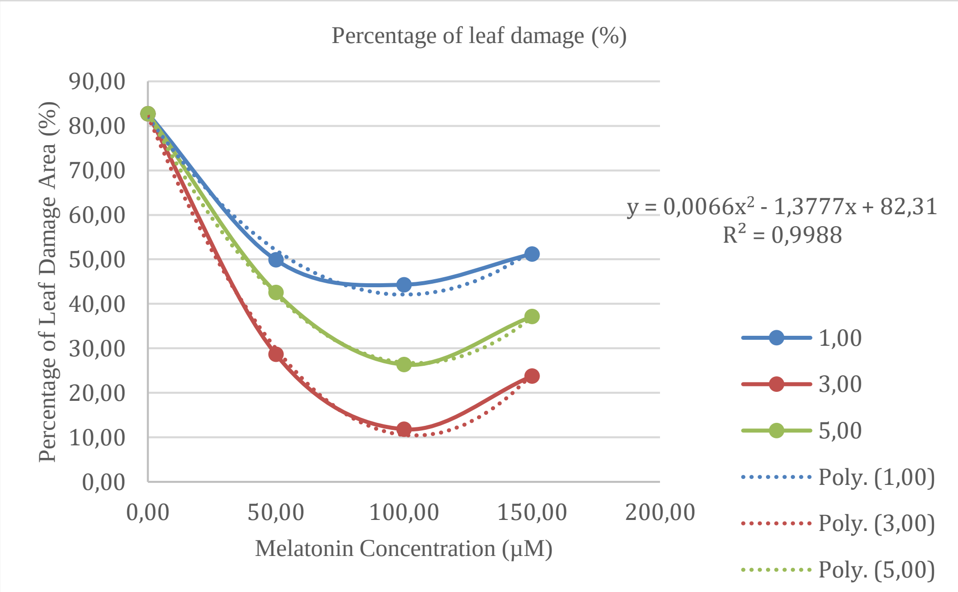


Fig. 7. Interaction between intervals application and melatonin concentration on leaf damage percentage

Fig. 7 presents data on the percentage of leaf damage as a response to melatonin application intervals at various concentrations. Overall, it can be seen that the application interval and melatonin concentration have a significant interaction in reducing the percentage of leaf damage. The 3-day application interval at various melatonin concentrations showed the highest results compared to other intervals. The treatment with a melatonin concentration of 0 µM (control) had the highest percentage of leaf damage at 82.73%. As the melatonin concentration increased, the percentage of leaf damage showed a significant decrease, then increased again at a concentration of 150 µM. The results of the orthogonal polynomial test of melatonin concentration showed a quadratic equation of y = 0.0066x2 – 1.3777 x + 82.31 and R^2 = 0.9998, indicating that the interaction between the application interval and melatonin concentration can affect the percentage of leaf damage caused by acid rain by 99.98%. Based on the quadratic equation, the optimal melatonin concentration was 104.37 µM with a leaf damage percentage of 10.41%, which was lower than the other treatment combinations. This interaction shows that melatonin can reduce toxicity in plant tissues under acid rain stress when applied at the appropriate application interval and concentration. High leaf damage percentages due to acid rain stress have the potential to disrupt plant physiology and reduce productivity. Leaf damage inhibits photosynthesis by reducing the leaf surface area available to capture sunlight energy, thereby decreasing photosynthetic efficiency.

Acid rain can increase ROS and MDA by 30.1% and 41.9%, respectively, causing chlorosis in rice seedlings, thereby disrupting the rate of photosynthesis. Analysis of most genes involved in chloroplast development and photosynthesis indicates disruption in the photosynthetic system located in the thylakoid membrane. Stress on plants in suboptimal growing conditions can reduce water content in leaf tissue, causing necrosis [37]. Optimal application of melatonin can increase cell water absorption capacity by modifying cell membrane and cell wall permeability, leading to an increase in the membrane stability index [38].

C. Percentage of stomatal damage (%/mm2)

The results of the orthogonal polynomial test of melatonin concentration treatment on the time of onset of necrosis are presented in Fig. 8. Fig. 8. shows the interaction between melatonin application intervals and melatonin concentration on the percentage of stomatal damage. The curve shows that the percentage of stomatal damage varies depending on the application interval and concentration of melatonin administered. In general, it can be seen that in the control treatment, stomatal damage is higher than in other treatments. Treatment at intervals of once every 3 days at various concentrations of melatonin was able to maintain stomatal condition, so that the percentage of stomatal damage decreased with increasing melatonin concentration to a certain point before experiencing a decrease in effectiveness.

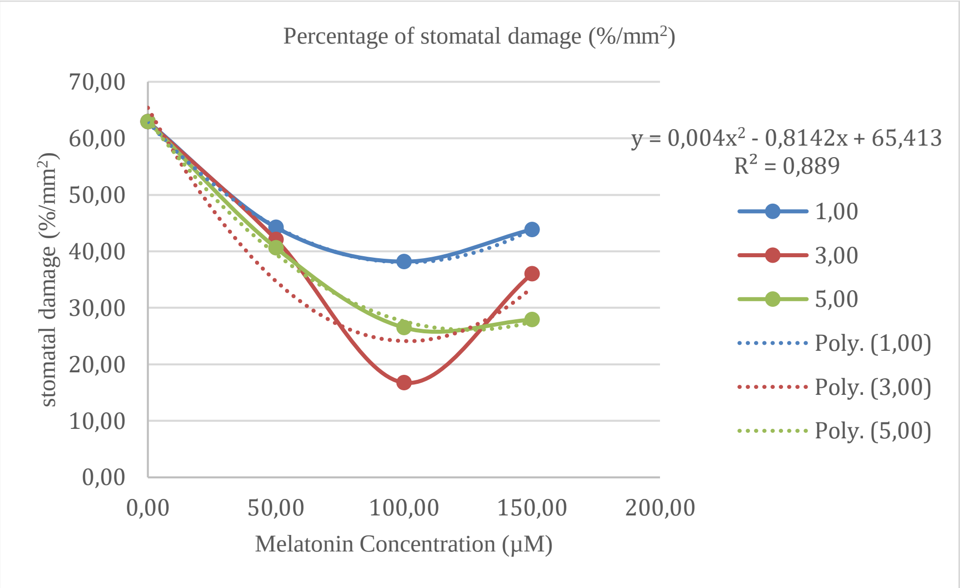


Fig. 8. Interaction between intervals application and melatonin concentration on the percentage of stomatal damage

The results of the orthogonal polynomial test of melatonin concentration showed a quadratic equation of y = 0.004x2 – 0.8142 x + 65.413 and R2 = 0.889, indicating that the interaction between the application interval and melatonin concentration can affect the percentage of stomatal damage due to acid rain stress by 88.9%. Based on the quadratic equation, the optimal melatonin concentration was 101.77 µM with a stomatal damage percentage of 23.98%, compared to the control of 62.96% and other treatment combinations. Melatonin is effective in plant tissues under acid rain stress when administered at the appropriate application interval and concentration.

Exogenous application of melatonin to leaves requires time to be absorbed by leaf tissue and translocated to target cells. The appropriate application interval and concentration allow sufficient time for the leaves to absorb and distribute melatonin throughout the tissue. Too frequent application is not expected to provide additional benefits and may cause unnecessary accumulation. Application at 3-day intervals can provide continuous but not excessive stimulation to the plant defense system. Low concentrations are less than optimal in providing acid rain stress resistance, while too high concentrations are thought to inhibit penetration into plant tissues.

Spraying melatonin at a concentration of 100 µM and applied every 3 days shows that melatonin, glutathione, and thiourea mediate plant protection from damage caused by acid rain, which shows stable membranes and protein structures, and increased antioxidant gene activity/expression. Research shows that exogenous melatonin is more efficient in increasing overall stress tolerance than glutathione and thiourea [25].

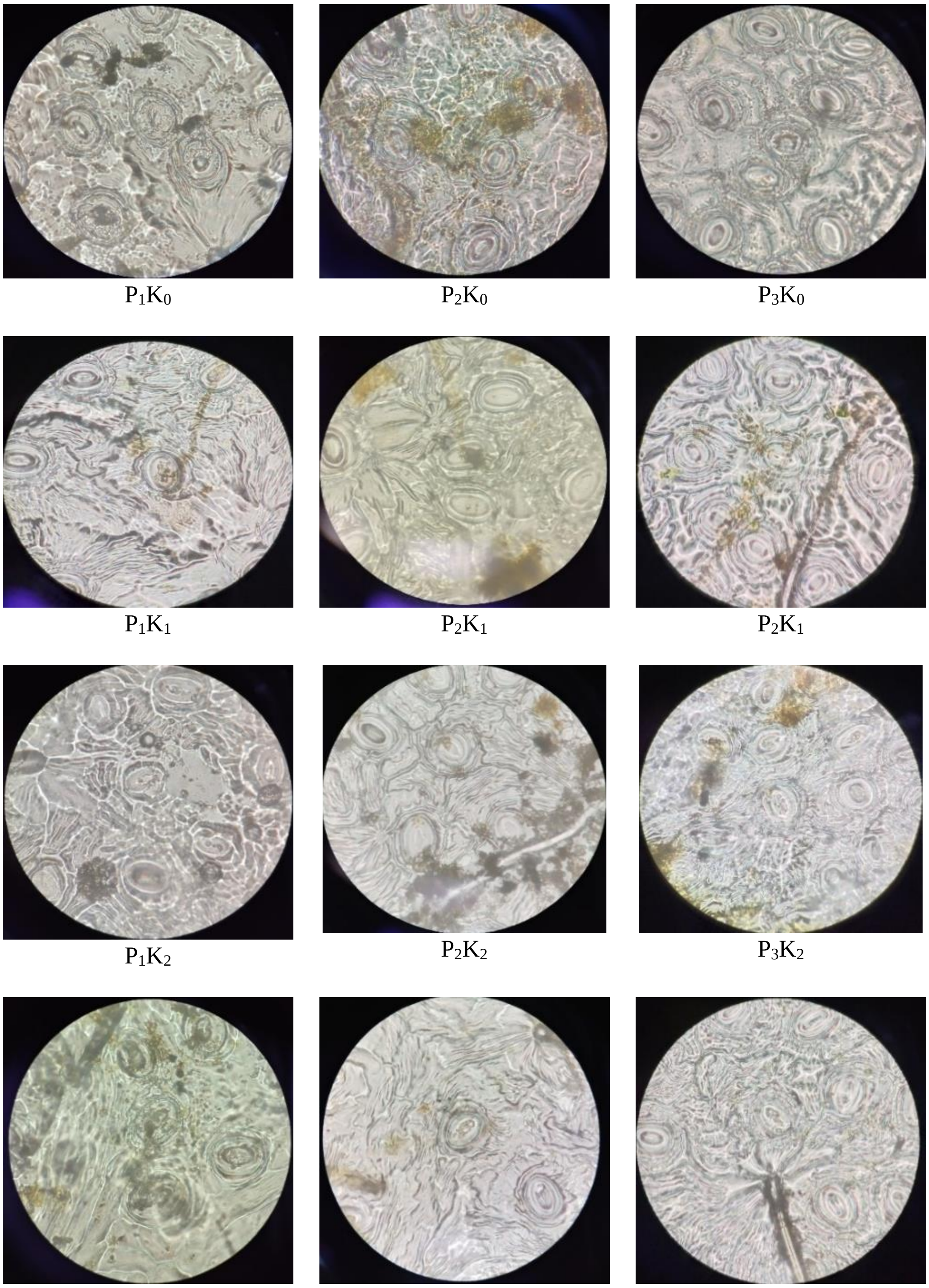


Fig. 9. Image of stomatal damage caused by acid rain stress

The results of the study indicate that acid rain with a pH of 3.5 affects the percentage of damage to stomata in terms of shape, structure, and cell integrity. Plants without melatonin (P1K0, P2K0, P3K0) and with a melatonin concentration of 150 µM at all melatonin application intervals (P1K3, P2K3, P3K3) exposed to acid rain caused damage to guard cells and neighboring cells, which form stomata. This damage is characterized by the appearance of spots on guard cells and neighboring cells, as well as the loss of turgor in stomata, preventing them from opening and closing properly. Epidermal cells around the stomata also shrink, causing the stomatal arrangement to become irregular and disorganized. Plants with the combination (P1K1, P2K1, P3K1, P1K2, P2K2, P3K2) show damage only in the guard cells and neighboring cells structurally, but still exhibit good cell turgor, so the stomata remain open.

Plants not exposed to acid rain show good stomatal and cell turgor contours, so the boundaries between each cell are clear and well-defined, while plants exposed to acid rain stress show microstructural damage, with most epidermal cells on the adaxial surface of the leaf exhibiting a shriveled structure, resulting in changes to the epidermal relief due to the formation of furrows between the wilted cells, and the guard cells of the stomata changed structurally, no longer showing the turgor aspect seen in the control treatment, and the outline of the stomatal pores also changed [39]. Although the number of stomata remained the same, as indicated by the stomatal density, there were no significant differences in intervals, concentration, and interaction. Damage to the structure and function of stomata due to acid rain can disrupt gas exchange and transpiration processes, potentially negatively impacting the growth and development of tea plants.

3.4 Anatomical appearance of the upper surface of leaves

Leaves are the first part affected by acid rain, which can cause morphological, anatomical, and photosynthetic pigment damage. Given that the cuticle layer on the upper surface of leaves is the first line of defense against acid deposition from acid rain, in-depth research on its anatomical appearance is crucial. An intact cuticle serves as a significant passive defense, reducing the risk of infection and disease spread in plants. Exposure to acid rain, which causes erosion and structural damage to the cuticle, results in cuticle damage, creating discontinuities and vulnerable entry points for pathogens to colonize plant tissues.

Acid rain is a form of precipitation with an acidic pH and high hydrogen ion concentration. Plants and soil act as absorbers and deposition sites for acid rain, with leaves, shoots, and roots being more sensitive to acidic pH, which affects soil composition—the primary medium for nutrient supply to plants and microflora. Acid rain solutions enter leaf tissues through the cuticle and stomata, causing significant corrosion effects on plants. Acid rain generally inhibits plant growth by inducing metabolic abnormalities in plants, such as photosynthesis, transpiration, and respiration [40]. The anatomical appearance of the upper surface of tea leaves under acid rain conditions is presented in Fig. 10.

Based on observations of the anatomical appearance of the upper leaf surface in the treatment combinations (P1K0, P2K0, P3K0, P1K1, P1K3, P2K3), damage to the cuticle was observed, with nearly complete erosion and a highly undulating surface appearance. This indicates that the cuticle structure on the upper surface layer was destroyed by exposure to acid rain. The treatment combinations (P1K2, P2K1, P3K1, P3K3) showed damage to the cuticle layer, with the surface exhibiting small waves in some areas. The treatment combinations (P2K2, P3K2) showed the cuticle shrinking unevenly in some areas.

Changes in leaf anatomy due to acid rain depend on acid concentration and exposure duration. Anatomical and biochemical damage begins to be detected at pH below 3.8. Damage is observed as necrotic spots and chlorosis ranging from yellow to brownish on the intercostal regions, trichome bases, and leaf margins. The cuticle undergoes changes when exposed to acidic solutions, including cuticle peeling and loss of epicuticular wax aggregates [41]. Water loss exceeding the roots' ability to absorb water leads to dehydration. The cuticle acts as a physical barrier preventing the penetration of pathogens such as fungi, bacteria, and viruses into plant tissues. Damage to the cuticle creates an entry point for pathogens to infect plant tissues.

Cuticle covers the outermost layer of epidermal cells and also acts as the first line of defense against environmental signals and biotic stress triggered by various pathogens and pests, such as fungi, bacteria, and insects [42]. Erosion of the leaf cuticle, the protective waxy layer on the leaf surface, significantly disrupts plant physiology. The cuticle's primary functions are to regulate water loss through transpiration and protect the plant from environmental stress. When the cuticle erodes due to factors like wind, rain, or sand particles, its integrity is compromised, leading to a drastic increase in transpiration. Plants lose more water than they can absorb, triggering water stress and stomata closure. While this stomatal closure reduces water loss, it also limits the intake of carbon dioxide, which directly inhibits the rate of photosynthesis and ultimately reduces plant growth and biomass production.

In addition to causing direct cuticle damage, exposure to acid rain stress that has already compromised the cuticle can penetrate leaf tissue more easily. The direct entry of acid can cause cellular damage, disrupt metabolic processes, and trigger tissue necrosis, further exacerbating the physiological stress on the plant. Furthermore, cuticle erosion diminishes the leaf's ability to defend against external threats. A damaged layer facilitates the invasion of pathogens and pest attacks, making plants more vulnerable to diseases and physical damage. The cuticle also plays a role in protecting against excessive UV radiation and extreme temperature fluctuations. When this layer erodes, that protection is reduced, potentially leading to cellular damage from UV or thermal stress. Overall, cuticle erosion creates a series of physiological challenges that weaken plants, hindering their ability to grow, reproduce, and survive in unfavorable environmental conditions.

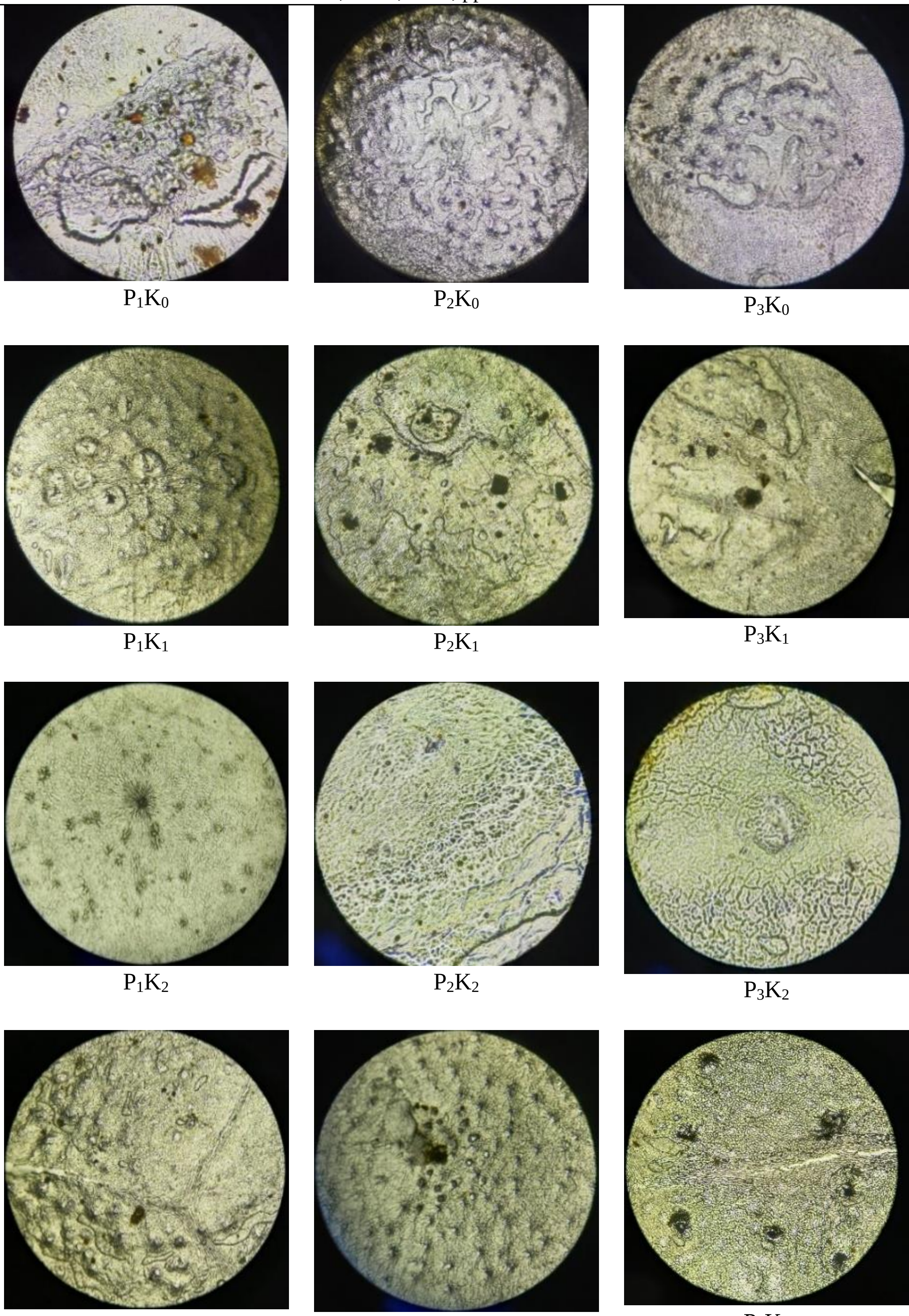


Fig. 10. Anatomical appearance of the upper surface of leaves

3.5 Anatomical appearance of cross-sectional leaf structure

Acid rain significantly affects plant growth and development. pH levels of 2.5 and 3.5 inhibit plant growth and cause visible damage to leaves, reducing the rate of photosynthesis and leaf transpiration. Low pH acid rain treatment has a stronger inhibitory effect on plant growth rates [43]. Cross-sectional leaf anatomy is presented in Fig. 11.

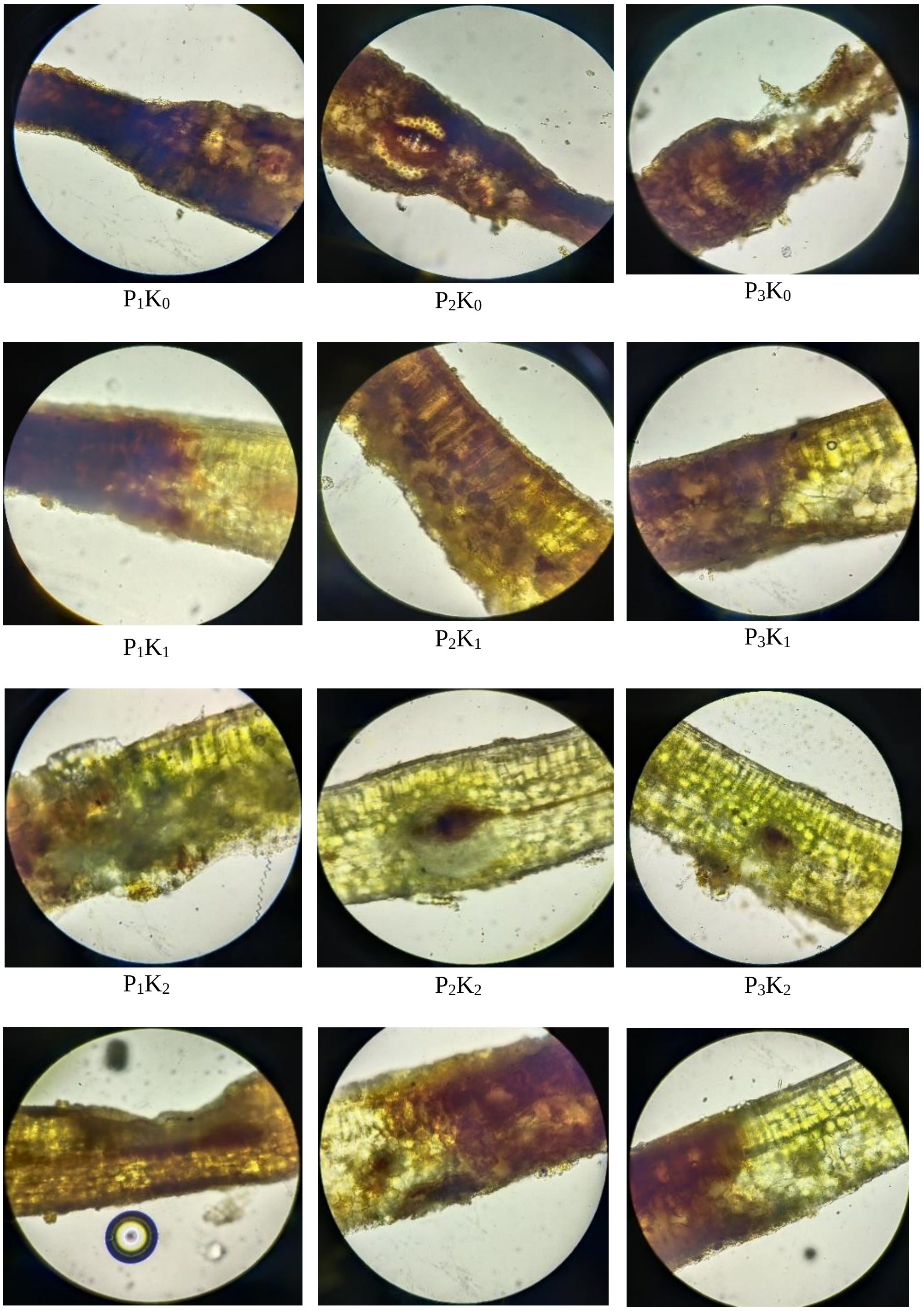


Fig. 11. Anatomical appearance of cross-sectional leaf structure

Acid rain with a pH of 3.5 in combination (P1K0, P2K0, P3K0, P1K3) causes damaged cells to turn very dark in color, with overall damage to the cuticle, upper epidermis, palisade parenchyma, vascular sheath, xylem and phloem transport bundles, coral tissue, and lower epidermis. The palisade parenchyma partially or completely collapses, resulting in cell shrinkage in the cross-section of the leaf, indicating cell drying due to exposure to acid rain. The treatment combinations (P1K1, P2K1, P3K1, P1K2, P2K3, P3K3) show damaged cells turning brownish in color, with damage occurring throughout the tissue but without cell thinning. The combinations (P2K2, P3K2) showed damage only to the vascular bundles and vascular bundles in the xylem and phloem tissues.

Cell and tissue damage in plant leaves has the potential to cause physiological disturbances in plants by disrupting the photosynthesis process. This leads to energy deficiency, inhibition of biomass formation, inhibition of growth and development, and can cause plant death. Acid rain can damage and even cause plant death by damaging leaf organs (waldsterben), including damage to the leaf surface wax and chlorophyll.

Dark color changes in leaf cells affected by acid rain indicate widespread damage leading to cell death and necrotic tissue, causing chlorosis, loss of cell structure, and changing leaf color to brown or black. The dark color originates from damaged and dead tissue, along with chlorophyll degradation. In line with the testing of leaf damage and chlorophyll percentages, the control plants showed the highest damage rate at 82.73%, with chlorophyll a, chlorophyll b, total chlorophyll, and chlorophyll ratio values of 2.48 mg/mL; 1.99 mg/mL; 4.46 mg/mL; and 1.26 mg/mL, respectively.

Xylem damage disrupts water flow, leading to wilting and loss of turgor in plants, and may interfere with nutrient absorption distribution. Phloem damage blocks the translocation of photosynthates to plant parts requiring them for growth, energy storage, and development. Xylem has the primary function of transporting water from the soil and the substances dissolved in it, while phloem transports photosynthetic products. The combination of xylem and phloem forms the vascular system throughout the plant body.

3.6 Photosynthetic pigments

Leaves are the primary organs of photosynthesis, containing chloroplasts that contain photosynthetic pigments, namely chlorophyll and carotenoids, which contribute to the process of capturing light energy. A cross-section of a leaf will show the distribution of chloroplasts and their pigments within the cells of the leaf mesophyll. The presence of chlorophyll and carotenoids together in chloroplasts highlights the crucial role of these pigments in the complex process of photosynthesis.

Chlorophyll pigments have maximum absorption in the blue and red bands of the visible light spectrum, reflecting and transmitting green light. Carotenoids are photosynthetic pigments with yellow, orange, and red colors visible in plants. Carotenoids function to expand the range of wavelengths that can contribute to photosynthesis by transferring absorbed energy to chlorophyll. The combination of various pigments makes the spectrum of sunlight energy absorption wider [14]. Based on the results of testing different intervals applications and concentrations of melatonin, the results of various photosynthetic pigments under acid rain stress conditions are presented in Fig. 12.

Chlorophyll pigment testing showed that the application of melatonin every 3 days at a concentration of 100 μM produced significantly higher values in chlorophyll a, total chlorophyll, and chlorophyll ratio tests compared to other treatments. Meanwhile, melatonin application every 3 days at a concentration of 150 μM showed the highest value in the chlorophyll b content test, with a difference of 1.16% compared to the treatment every 3 days at a concentration of 100 μM. The lowest chlorophyll a and total chlorophyll levels were obtained in the control group, while in the chlorophyll b level test, the lowest value was obtained in the treatment with an interval of once every 5 days and a concentration of 150 μM, with a difference of 9.93% compared to the control. The lowest chlorophyll ratio was recorded in the treatment with an interval of once a day and a concentration of 50 μM, with a difference of 6.90% compared to the control. The results of photosynthetic pigment testing showed a decrease in chlorophyll a, chlorophyll b, total chlorophyll, chlorophyll ratio, and total carotene by 65.69%, 50.66%, 58.69%, 41.91%, and 64.58%, respectively, when comparing the treatment with the highest and lowest concentrations under acid rain stress. Research shows that the application of melatonin at the right intervals and effective concentration is effective in preventing chlorophyll degradation in tea plants under acid rain conditions, and a stable amount is thought to be able to maintain the optimal rate of plant photosynthesis.

Acid rain triggers cadmium (Cd) reactions in soil, with anion composition and acid rain pH being two key factors influencing Cd formation effects [44]. Cd causes significant changes in membrane function by inducing lipid peroxidation and disrupting chloroplast metabolism by inhibiting chlorophyll biosynthesis and reducing the activity of enzymes involved in CO_2 fixation. The factors that determines optimal photosynthesis is the amount of chlorophyll contained in the leaves. Chlorophyll content positively correlates with photosynthesis yield; the more chlorophyll present in the leaves, the higher the photosynthesis yield.

Testing of the chlorophyll a/b ratio showed that the application of exogenous melatonin every 3 days at a concentration of 100 μM showed the highest result, namely 2.01 mg/mL. Melatonin plays a role in mitigating the negative effects of acid rain, so that proper application is able to maintain the chlorophyll ratio at a higher level compared to other treatments, but melatonin is thought to be limited in controlling acid rain toxicity at extreme acidity levels at pH 3.5, so that the chlorophyll ratio achieved is still below optimal conditions. The study results indicate that acid rain with a pH of 3.5 significantly reduces leaf chlorophyll ratio, disrupting the balance between chlorophyll a and b, which is crucial for effective light spectrum absorption. The application of melatonin every 3 days at a concentration of 100 µM on plants experiencing acid rain stress showed the highest chlorophyll ratio, namely 2.01 mg/ml. The results showed that the chlorophyll ratio decreased by 41.91% when compared to the treatment with the highest and lowest amounts of acid rain stress.

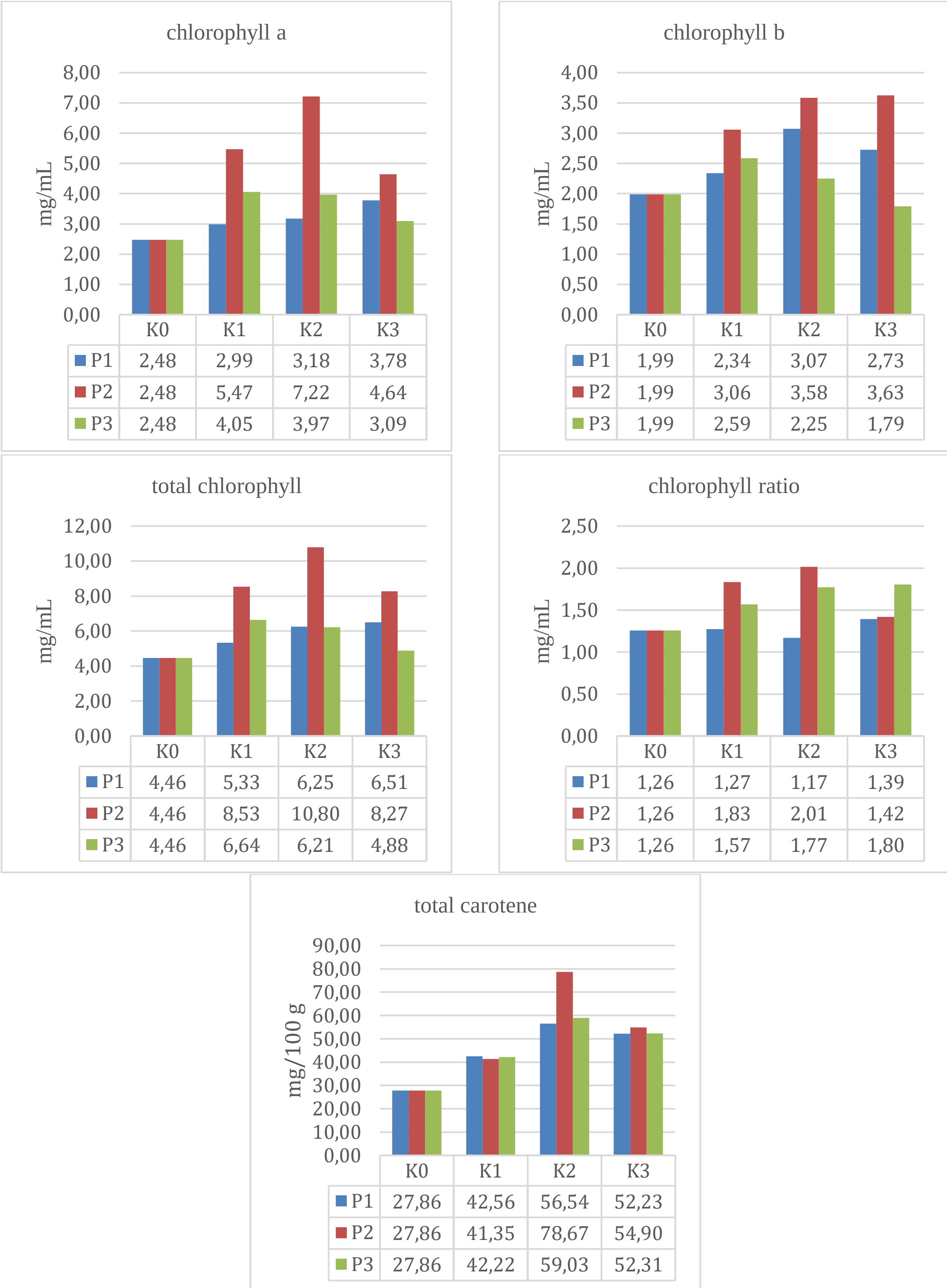


Fig. 12. Photosynthetic pigments

An optimal chlorophyll ratio is crucial in the photosynthesis process because chlorophyll b plays a key role in capturing light energy and then transporting that energy to chlorophyll a, which is the center of the photosynthesis reaction. An suboptimal chlorophyll ratio, as observed in the control plants, may disrupt the efficiency of energy transport from chlorophyll b to chlorophyll a, thereby reducing overall photosynthesis rates because the captured light energy cannot be fully utilized for photosynthesis. The chlorophyll a/b ratio in plants ranges from 1.5 to 4.7, with an average value of 3.1. High levels of chlorophyll, particularly chlorophyll a, contribute to the green coloration of plants.

The leaf chlorophyll content is an important indicator of direct damage caused by acid rain on leaves and leads to a decrease in plant productivity. The results indicate that acid rain substantially reduces leaf chlorophyll content by 6.71% per unit of pH across 67 plant species [45]. Chlorophyll content in leaf tissue fundamentally acts as the primary pigment responsible for the photosynthesis process, converting light energy into chemical energy essential for plant growth and development. A significant decrease in chlorophyll concentration reflects degradation of photosynthetic activity within chloroplasts, which will result in reduced plant productivity due to diminished ability to absorb sunlight for photosynthesis.

3.7 Implications and explanations of research findings

This study has significant practical implications for the agricultural sector, particularly tea cultivation under acid rain stress conditions. The finding that the application of melatonin at an interval of 3 days and a concentration of 100 µM can optimally delay necrosis, reduce the percentage of leaf and stomatal damage, and increase photosynthetic pigment levels (chlorophyll a, total chlorophyll, chlorophyll ratio, and total carotene) offers a clear and measurable mitigation protocol. This means that tea farmers now have science-based guidance for applying melatonin as a proactive strategy in dealing with acid rain stress. By adopting the findings of this study, it is hoped that tea plant productivity can be maintained, economic losses due to acid rain stress can be minimized, and the sustainability of agricultural practices in acid rain-prone areas can be improved. The success in tea plants also opens opportunities for further exploration in other plant species facing similar stress.

The effectiveness of the 3-day interval and 100 µM concentration of melatonin in this study can be scientifically explained through the multifunctional role of melatonin as a pleiotropic molecule. The 3-day interval most likely reflects the optimal period for maintaining effective exogenous melatonin levels in plant tissues after metabolism or transport, ensuring continuous protection without causing saturation or waste. The 100 µM concentration, on the other hand, is likely within the range of concentrations that are bioactive enough to trigger adaptive physiological responses. Melatonin is known to induce changes in gene expression related to abiotic stress responses, strengthen the plant antioxidant system, reduce the accumulation of ROS and RNS, maintain cellular integrity, including chloroplasts and cell walls, and restore and enhance photosynthetic pigment synthesis. The interaction between internal and concentration suggests that the effectiveness of melatonin is highly dependent on its availability and appropriate levels within a specific time frame, which collectively increase the tolerance of tea plants to oxidative stress and cellular damage caused by acid rain.

3.8 Strengths and limitations

This study addresses a highly relevant and urgent issue, namely the mitigation of the effects of acid rain on tea plants, which are an important commodity in Indonesia, by analyzing the optimal internal and external conditions for increasing the tolerance of tea plants to acid rain stress. This study observed various important parameters that reflect plant responses to stress, including morphological aspects (time of necrotic appearance, percentage of leaf damage area), anatomical (stomatal density, percentage of stomatal damage, severity of stomatal damage, leaf anatomical integrity), and photosynthetic pigment physiology (chlorophyll a, b, total chlorophyll, chlorophyll ratio, and total carotenoids). The results clearly identified a 3-day application interval and a concentration of 100 µM as the most effective combination for mitigating acid rain damage, providing direct recommendations that can be applied in agricultural practice.

Although acid rain simulations allow for controlled conditions, these conditions may not fully replicate the complexity of natural acid rain in the field, which varies in chemical composition, intensity, and frequency. Although the parameters observed were quite comprehensive, the study did not cover several other physiological or biochemical aspects that may be relevant, such as the activity of specific antioxidant enzymes (e.g., catalase, superoxide dismutase, ascorbate peroxidase), osmolyte levels, or the expression of stress-related genes, which could provide a deeper understanding of the protective mechanism of melatonin. This study was conducted on the Gambung 7 tea clone, and it is suspected that the response to melatonin and acid rain may vary between tea clones, so the results may not be directly generalizable.

4. CONCLUSION

The 3-day interval showed longer necrotic results, with an average of 8.5 days after acid rain, lower leaf damage area compared to the 1- and 5-day intervals (36.74%), and stomatal damage that was not significantly different from the 5-day interval. A concentration of 119.85 µM showed a longer onset of necrosis, specifically

10 days after acid rain, with a leaf damage area of 26.15% at a concentration of 104 µM, and stomatal damage of 29.24% at a concentration of 109.29 µM. There was an interaction between the application interval and melatonin concentration, which increased the effectiveness and mechanism of improving tea plant performance under acid rain stress. The 1-day and 3-day intervals with a concentration of 100 µM showed the lowest damage to the upper surface and cross-section anatomy of the leaves. The 3-day interval with a concentration of 100 µM produced the highest amount of chlorophyll a, total chlorophyll, chlorophyll ratio, and total carotene, namely 7.22 mg/mL; 10.80 mg/mL; 2.01 mg/mL; and 78.76 mg/100 g. Melatonin at a concentration of 150 µM every 3 days showed the highest amount of chlorophyll b, namely 3.58 mg/mL. We hope that further research will focus on the effectiveness of melatonin in improving the performance of tea plants under other types of abiotic stress or a combination of various stresses. Further research on physiological or biochemical aspects, specific antioxidant enzyme activity, and stress-related gene expression is certainly important to provide a deeper understanding of the protective mechanism of melatonin under acid rain stress conditions. In addition, it is necessary to investigate whether melatonin and acid rain can vary between tea clones, so that the results can be directly applied to all types of tea plants. It is also important to investigate innovations to increase endogenous melatonin production in tea plants as a sustainable strategy to improve plant resistance to unfavorable environmental conditions.

Author Contribution

All authors contributed equally to the main contributor to this paper. All authors have read and agreed to the published version of the manuscript.

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Conflict of Interest

The authors declare no conflict of interest.

5. REFERENCES

[1] I. Ahmad, G. Zhu, G. Zhou, J. Liu, M. U. Younas, and Y. Zhu, 'Melatonin Role in Plant Growth and Physiology under Abiotic Stress', Int. J. Mol. Sci., vol. 24, no. 10, p. 8759, May 2023, doi: https://doi.org/10.3390/ijms24108759.

[2] C. Zhang et al., 'Physiological and biochemical responses of tea seedlings (Camellia sinensis) to simulated acid rain conditions', Ecotoxicol. Environ. Saf., vol. 192, p. 110315, Apr. 2020, doi: https://doi.org/10.1016/j.ecoenv.2020.110315.

[3] V. M. Rodríguez-Sánchez, U. Rosas, G. Calva-Vásquez, and E. Sandoval-Zapotitla, 'Does Acid Rain Alter the Leaf Anatomy and Photosynthetic Pigments in Urban Trees?', Plants, vol. 9, no. 7, p. 862, Jul. 2020, doi: https://doi.org/10.3390/plants9070862.

[4] 'Morphology and Anatomy', in Postharvest Physiology and Biochemistry of Fruits and Vegetables, Elsevier, 2019, pp. 113-130, https://doi.org/10.1016/B978-0-12-813278-4.00006-3.

[5] K. Sakoda, W. Yamori, T. Shimada, S. S. Sugano, I. Hara-Nishimura, and Y. Tanaka, 'Higher Stomatal Density Improves Photosynthetic Induction and Biomass Production in Arabidopsis Under Fluctuating Light', Front. Plant Sci., vol. 11, Oct. 2020, doi: https://doi.org/10.3389/fpls.2020.589603.

[6] T. N. Buckley, 'How do stomata respond to water status?', New Phytol., vol. 224, no. 1, pp. 21-36, Oct. 2019, doi: https://doi.org/10.1111/nph.15899.

[7] C. Bowsher and A. K. Tobin, Plant biochemistry, Second edition. Boca Raton, FL: CRC Press, 2021, https://doi.org/10.1201/9781003137986.

[8] M. P. Johnson, 'Photosynthesis', Essays Biochem., vol. 60, no. 3, pp. 255-273, Oct. 2016, doi: https://doi.org/10.1042/EBC20160016.

[9] S. K. Bose and P. Howlader, 'Melatonin plays multifunctional role in horticultural crops against environmental stresses: A review', Environ. Exp. Bot., vol. 176, p. 104063, Aug. 2020, doi: https://doi.org/10.1016/j.envexpbot.2020.104063.

[10] H. Sati, A. V. Chinchkar, P. Kataria, and S. Pareek, 'Melatonin: A potential abiotic stress regulator', Plant Stress, vol. 10, p. 100293, Dec. 2023, doi: https://doi.org/10.1016/j.stress.2023.100293.

[11] B. Debnath et al., 'Exogenous Melatonin Mitigates Acid Rain Stress to Tomato Plants through Modulation of Leaf Ultrastructure, Photosynthesis and Antioxidant Potential', Molecules, vol. 23, no. 2, p. 388, Feb. 2018, doi: https://doi.org/10.3390/molecules23020388.

[12] X. Tan, J. Huang, L. Lin, and Q. Tang, 'Exogenous Melatonin Attenuates Cd Toxicity in Tea (Camellia sinensis)', Agronomy, vol. 12, no. 10, p. 2485, Oct. 2022, doi: https://doi.org/10.3390/agronomy12102485.
[13] C. Zhang et al., 'Physiological and biochemical responses of tea seedlings (Camellia sinensis) to simulated acid rain conditions', Ecotoxicol. Environ. Saf., vol. 192, p. 110315, Apr. 2020, doi: https://doi.org/10.1016/j.ecoenv.2020.110315.
[14] C. Bowsher and A. K. Tobin, Plant biochemistry, 2nd ed. Abingdon, United Kingdom: CRC press, Taylor & Francis group, 2021, https://doi.org/10.1201/9781003137986.
[15] C. Li, D.-X. Tan, D. Liang, C. Chang, D. Jia, and F. Ma, 'Melatonin mediates the regulation of ABA metabolism, free-radical scavenging, and stomatal behaviour in two Malus species under drought stress', J. Exp. Bot., vol. 66, no. 3, pp. 669-680, Feb. 2015, doi: https://doi.org/10.1093/jxb/eru476.
[16] Y. Cui, M. He, D. Liu, J. Liu, J. Liu, and D. Yan, 'Intercellular Communication during Stomatal Development with a Focus on the Role of Symplastic Connection', Int. J. Mol. Sci., vol. 24, no. 3, p. 2593, Jan. 2023, doi: https://doi.org/10.3390/ijms24032593.
[17] M. Haworth, G. Marino, A. Materassi, A. Raschi, C. P. Scutt, and M. Centritto, 'The functional significance of the stomatal size to density relationship: Interaction with atmospheric [CO2] and role in plant physiological behaviour', Sci. Total Environ., vol. 863, p. 160908, Mar. 2023, doi: https://doi.org/10.1016/j.scitotenv.2022.160908.
[18] A. Ramos, S. Almeida, L. Ganço, P. Costa, and A. Pereira, 'Impact of Acid Rain with Different pH Values in Monstera deliciosa Plants', in The 6th International Congress of CiiEM—Immediate and Future Challenges to Foster One Health, Basel Switzerland: MDPI, Aug. 2023, p. 21. doi: https://doi.org/10.3390/msf2023022021.
[19] Z. J. Ding, Y. Z. Shi, G. X. Li, N. P. Harberd, and S. J. Zheng, 'Tease out the future: How tea research might enable crop breeding for acid soil tolerance', Plant Commun., vol. 2, no. 3, p. 100182, May 2021, doi: https://doi.org/10.1016/j.xplc.2021.100182.
[20] H.-E. Sadia, F. Jeba, Md. Z. Uddin, and A. Salam, 'Sensitivity study of plant species due to traffic emitted air pollutants (NO2 and PM2.5) during different seasons in Dhaka, Bangladesh', SN Appl. Sci., vol. 1, no. 11, Nov. 2019, doi: https://doi.org/10.1007/s42452-019-1421-4.
[21] G. C. Andrade, L. N. Castro, and L. C. D. Silva, 'Micromorphological alterations induced by simulated acid rain on the leaf surface of Joannesia princeps Vell. (Euphorbiaceae)', Ecol. Indic., vol. 116, p. 106526, Sep. 2020, doi: https://doi.org/10.1016/j.ecolind.2020.106526.
[22] Z. Guo et al., 'Effects of Heavy Metals on Stomata in Plants: A Review', Int. J. Mol. Sci., vol. 24, no. 11, p. 9302, May 2023, doi: https://doi.org/10.3390/ijms24119302.
[23] C. Zhang et al., 'Comprehensive transcriptome profiling of tea leaves (Camellia sinensis) in response to simulated acid rain', Sci. Hortic., vol. 272, p. 109491, Oct. 2020, doi: https://doi.org/10.1016/j.scienta.2020.109491.
[24] B. Debnath, M. Li, S. Liu, T. Pan, C. Ma, and D. Qiu, 'Melatonin-mediate acid rain stress tolerance mechanism through alteration of transcriptional factors and secondary metabolites gene expression in tomato', Ecotoxicol. Environ. Saf., vol. 200, p. 110720, Sep. 2020, doi: https://doi.org/10.1016/j.ecoenv.2020.110720.
[25] R. Xalxo and S. Keshavkant, 'Melatonin, glutathione and thiourea attenuates lead and acid rain-induced deleterious responses by regulating gene expression of antioxidants in Trigonella foenum graecum L.', Chemosphere, vol. 221, pp. 1-10, Apr. 2019, doi: https://doi.org/10.1016/j.chemosphere.2019.01.029.
[26] H. Sati, A. V. Chinchkar, P. Kataria, and S. Pareek, 'Melatonin: A potential abiotic stress regulator', Plant Stress, vol. 10, p. 100293, Dec. 2023, doi: https://doi.org/10.1016/j.stress.2023.100293.
[27] B. Debnath, M. Li, S. Liu, T. Pan, C. Ma, and D. Qiu, 'Melatonin-mediate acid rain stress tolerance mechanism through alteration of transcriptional factors and secondary metabolites gene expression in tomato', Ecotoxicol. Environ. Saf., vol. 200, p. 110720, Sep. 2020, doi: https://doi.org/10.1016/j.ecoenv.2020.110720.
[28] E.-U. Liu and C.-P. Liu, 'Effects of Simulated Acid Rain on the Antioxidative System in Cinnamomum philippinense Seedlings', Water. Air. Soil Pollut., vol. 215, no. 1-4, pp. 127-135, Feb. 2011, doi: https://doi.org/10.1007/s11270-010-0464-3.
[29] M. Faizan et al., 'Melatonin and its cross-talk with other signaling molecules under abiotic stress', Plant Stress, vol. 11, p. 100410, Mar. 2024, doi: https://doi.org/10.1016/j.stress.2024.100410.
[30] N. Zhang et al., 'Roles of melatonin in abiotic stress resistance in plants', J. Exp. Bot., vol. 66, no. 3, pp. 647-656, Feb. 2015, doi: https://doi.org/10.1093/jxb/eru336.
[31] S. Cherono et al., 'Exogenous Application of Melatonin Improves Drought Tolerance in Coffee by Regulating Photosynthetic Efficiency and Oxidative Damage', J. Am. Soc. Hortic. Sci., vol. 146, no. 1, pp. 24-32, Jan. 2021, doi: https://doi.org/10.21273/JASHS04964-20.
[32] X. Tan, J. Huang, L. Lin, and Q. Tang, 'Exogenous Melatonin Attenuates Cd Toxicity in Tea (Camellia sinensis)', Agronomy, vol. 12, no. 10, p. 2485, Oct. 2022, doi: https://doi.org/10.3390/agronomy12102485.
[33] S. Ahmad et al., 'Foliar application of melatonin delay leaf senescence in maize by improving the antioxidant defense system and enhancing photosynthetic capacity under semi-arid regions', Protoplasma, vol. 257, no. 4, pp. 1079-1092, Jul. 2020, doi: https://doi.org/10.1007/s00709-020-01491-3.
[34] M. B. Arnao and J. Hernández-Ruiz, 'Melatonin as a Chemical Substance or as Phytomelatonin Rich-Extracts for Use as Plant Protector and/or Biostimulant in Accordance with EC Legislation', Agronomy, vol. 9, no. 10, p. 570, Sep. 2019, doi: https://doi.org/10.3390/agronomy9100570.
[35] V. Martinez et al., 'Tolerance to Stress Combination in Tomato Plants: New Insights in the Protective Role of Melatonin', Molecules, vol. 23, no. 3, p. 535, Feb. 2018, doi: https://doi.org/10.3390/molecules23030535.

[36] B. Debnath et al., 'Exogenous Melatonin Mitigates Acid Rain Stress to Tomato Plants through Modulation of Leaf Ultrastructure, Photosynthesis and Antioxidant Potential', Molecules, vol. 23, no. 2, p. 388, Feb. 2018, doi: https://doi.org/10.3390/molecules23020388.
[37] Y. Zhang, F. Yang, Y. Wang, Y. Zheng, and J. Zhu, 'Effects of Acid Rain Stress on the Physiological and Biochemical Characteristics of Three Plant Species', Forests, vol. 14, no. 5, p. 1067, May 2023, doi: https://doi.org/10.3390/f14051067.
[38] H. Muhammad Tayyab Khan, R. Mukhtar Balal, Z. Hussain, S. Ayyaz Javed, M. Tauseef Jaffar, and A. Abdullah Alsahli, 'Exogenous application of melatonin mitigate the heat stress in different tomato (Solanum lycopersicum L.) cultivars', J. King Saud Univ. - Sci., vol. 36, no. 3, p. 103086, Mar. 2024, doi: https://doi.org/10.1016/j.jksus.2023.103086.
[39] G. C. Andrade, L. N. Castro, and L. C. D. Silva, 'Micromorphological alterations induced by simulated acid rain on the leaf surface of Joannesia princeps Vell. (Euphorbiaceae)', Ecol. Indic., vol. 116, p. 106526, Sep. 2020, doi: https://doi.org/10.1016/j.ecolind.2020.106526.
[40] N. Lal, 'Effects of Acid Rain on Plant Growth and Development'.
[41] V. M. Rodríguez-Sánchez, U. Rosas, G. Calva-Vásquez, and E. Sandoval-Zapotitla, 'Does Acid Rain Alter the Leaf Anatomy and Photosynthetic Pigments in Urban Trees?', Plants, vol. 9, no. 7, p. 862, Jul. 2020, doi: https://doi.org/10.3390/plants9070862.
[42] G. C. Arya, S. Sarkar, E. Manasherova, A. Aharoni, and H. Cohen, 'The Plant Cuticle: An Ancient Guardian Barrier Set Against Long-Standing Rivals', Front. Plant Sci., vol. 12, Jun. 2021, doi: https://doi.org/10.3389/fpls.2021.663165.
[43] X. Shu, K. Zhang, Q. Zhang, and W. Wang, 'Ecophysiological responses of Jatropha curcas L. seedlings to simulated acid rain under different soil types', Ecotoxicol. Environ. Saf., vol. 185, p. 109705, Dec. 2019, doi: https://doi.org/10.1016/j.ecoenv.2019.109705.
[44] H. Zhu, C. Wu, J. Wang, and X. Zhang, 'The effect of simulated acid rain on the stabilization of cadmium in contaminated agricultural soils treated with stabilizing agents', Environ. Sci. Pollut. Res., vol. 25, no. 18, pp. 17499-17508, Jun. 2018, doi: https://doi.org/10.1007/s11356-018-1929-y.
[45] E. Du, D. Dong, X. Zeng, Z. Sun, X. Jiang, and W. De Vries, 'Direct effect of acid rain on leaf chlorophyll content of terrestrial plants in China', Sci. Total Environ., vol. 605-606, pp. 764-769, Dec. 2017, doi: https://doi.org/10.1016/j.scitotenv.2017.06.044.