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Original Article

The effect of different light intensities on the anatomical and chemical characteristics of the aquatic plant of *Azolla pinnata*

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Abstract: *Azolla pinnata* is one of the important aquatic plants used as feed for fish and poultry. The current work aimed to study the effect of varying lighting intensities on the anatomical characteristics and chemical compounds of *A. pinnata*. The experiment was conducted to culture *Azolla* under different light intensities of 0, 15,000, 10,000, 5,000, and 2,000 lux. The results showed that differences in light intensities affected most of the anatomical characteristics of *A. pinnata*. Increases and decreases in light intensity led to variations in the thickness of the vertical walls of the epidermis and the cuticle layer and in the dimensions of the epidermal cells on the upper and lower surfaces. Additionally, these changes affected the thickness and dimensions of the mesophyll tissues. A decrease in light intensity to 2,000 lux also impacted the mesophyll, leading to the rupture of its cells. Furthermore, primary pits were observed on the upper and lower surfaces, along with an abundance of *Anabaena azolla* algal cells on the lower surface, especially when the light intensity decreased to 5,000 and 2,000 lux. On the other hand, the increase and decrease in light intensity affected the chemical compounds, resulting in the appearance and disappearance of some secondary compounds. Notably, two phenolic compounds were identified: vanillin acetate and phenol, 2,2'-methylenebis[6-(1,1-dimethyl ethyl)-4-methyl-]. Some compounds were shared among the different light treatments, with varying concentrations of the same compound compared to the control treatment.

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Introduction

Azolla pinnata L., belonging to the Azollaceae family, is known as water fern, water mosquito, or duck fern. It is an aquatic plant that floats on the surface of freshwater bodies or ponds (Mahmood et al., 2020). Banach et al. (2019) stated that it is a C3 plant characterized by its ability to reduce CO_2 through the participation of the enzyme rubisco. It is native to tropical, subtropical, and temperate regions, including Asia, Africa, and North and South America. *Azolla microphylla* is found in tropical regions, while *A. nilotica* is found in East Africa, from Sudan to Mozambique, and *A. pinnata* is more widespread in Asian regions (Rashad, 2021; Adabembe et al., 2022).

In addition, the plant typically measures 1-2.5 cm in length, although some species may reach 15 cm or more. It consists of a very short, branched stem from which fibrous, hairy roots usually emerge from any point and hang beneath the water's surface. Its leaves are small, arranged alternately, and have two lobes. The dorsal lobe is usually thick and contains chlorophyll, while the ventral lobe is thin, slightly larger than the dorsal lobe, and is characterized by its lack of color. This structure helps the plant float on the water's surface (Al-Araji, 2020).

Light is an essential resource for ecosystems, whether in aquatic or terrestrial environments, as it represents the primary energy source for most living organisms, especially plants. The increase, decrease, or absence of light affects many physiological and anatomical processes in plants and the formation of chemical substances. It also influences the growth and development of plants, in addition to its effects on plant adaptation and the synthesis of photosynthetic

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pigments (Markos and Asrat, 2020). Washeeh (2018) stated that most of these processes, along with the anatomical and chemical characteristics of aquatic plants, are influenced by various environmental factors, including light intensity, the effects of which are also reflected in the distribution of plants in different environments.

Studies have shown that the response of *Azolla* species to light varies, with some plants, such as *Azolla microphylla*, preferring high light-conditions. In contrast, the opposite is true for *A. caroliniana* and *A. pinnata*, which require only 50% of the light intensity. An increase above this percentage negatively affects these two species' physiological and chemical reactions. Van Kempen et al. (2016) stated that light plays a fundamental role in the reactions within the plant body, which is reflected in the chemical content of the *Azolla* plant, particularly in phenols and terpenes. Washeeh et al. (2024) also pointed out the importance of chemical compounds, especially phenolic compounds, in aquatic plants, as they serve as a defense mechanism against various environmental factors, such as light, that can lead to photo-oxidation. Therefore, these compounds protect plant cells, tissues, and membranes from various photo-oxidative damages.

On the other hand, many studies have indicated the importance of aquatic plants, including *Azolla*, in various fields. It is a rich source of protein, amino acids, vitamins, and minerals, making it a healthy food option for humans and animals. These plants are also used in raising fish, poultry, and livestock as an alternative to traditional feed, which improves the quality of meat, eggs, and milk (Kumar et al., 2020). Additionally, these plants are rich in nutrients and antioxidants, enhancing immune system health and reducing blood cholesterol levels while improving the digestive system (Kumar, 2021; Adzman et al., 2022).

Beyond its economic importance, *Azolla* has been utilized in developing countries to promote regional development and provide job opportunities (Acharya et al., 2015). The chemical extracts of these plants have also been used as insecticides to control weeds in wheat fields, particularly invasive species (Gul et al., 2017; Chowdhury, 2022). Therefore, the current study, the first of its kind in the Diwaniyah Governorate, Iraq, aims to determine how different lighting intensities affect the anatomical and chemical characteristics of the aquatic plant *A. pinnata*.

Materials and Methods

An experiment on the growth of *A. pinnata* was conducted in glass basins measuring 50x50x40 cm in February 2023. The aquaria were thoroughly washed with chlorine-free water to eliminate impurities and suspended microorganisms. The plants were then exposed to high and low light intensities using light-emitting diodes (LEDs) set at 15,000, 10,000, 5,000, and 2,000 lux, and these treatments were compared with a control group. The lighting system operated for 14 hours daily in the Biology Sciences Laboratory at Al-Qadisiyah University, College of Education, for 21 days. After this period, samples were collected and examined to assess the effects of different light intensities on anatomical and chemical characteristics.

Anatomical studies methods: *Azolla* leaves were dissected using two methods: (1) According to Washeeh (2018), the method was employed for each light treatment to prepare the upper and lower epidermis. This involved scraping the epidermis using a scalpel and pointed forceps. The chlorophyll pigment was removed from the epidermis using a 2% NaOH solution for 5 minutes. Afterward, the epidermis was washed several times with distilled water to remove the solution. The epidermis samples were stained with safranin and glycerin after being washed with alcohol. Finally, the epidermis was transferred for examination under a light microscope. (2) Anatomy of cross-sections: The rotating microtome was used to dissect cross-sections of *Azolla* leaves treated with different light intensities in the Biology Sciences Laboratory at the University of Baghdad. This was done after applying several methods to prepare the samples for dissection, as follows:

Killing and fixation: 15 ml of formalin acetic acid alcohol (FAA) was used, and the plant samples were left in the solution for 24 hours at laboratory

temperature.

Washing and dehydration: The plant samples were washed twice with 70% ethyl alcohol to remove traces of the fixative and were preserved in alcohol at the same concentration. The *Azolla* leaf samples were then passed through an ascending series of alcohol concentrations (80, 90, and 96%) for one hour at each concentration, followed by immersion in absolute ethyl alcohol for 30 minutes to eliminate water from the plant sections.

Clearing and infiltration: The *Azolla* samples were sequentially passed through a mixture of absolute ethyl alcohol and xylene in 1:3, 1:1, and 3:1 volume ratios, followed by pure xylene for one hour. The xylene was then replaced with fresh, pure xylene for 3-4 minutes. After that, half of the xylene containing the plant samples was removed and replaced with liquid paraffin in an oven at a temperature of 55-60°C for 48 hours. This was followed by replacing it with another batch of wax and leaving it in the oven overnight.

Embedding and mounting: After removing the wax, the plant specimens were embedded in paraffin using cardboard. The *Azolla* specimens were then stained and prepared for dissection using the microtome device, followed by examination under an electron microscope.

Preparation of chemical extracts of *Azolla*: Plant extracts for chemical analysis were prepared using the GSMS device, following the method of Washeeh et al. (2024). All plant samples were thoroughly washed with distilled water to remove dust and impurities, then left to dry at laboratory temperature away from light for two days. The samples were ground in an electric grinder for 11 minutes to obtain a smooth mixture. One gram of crushed *Azolla* leaves was extracted for each light treatment separately and dissolved in 10 ml of 99% methanol with continuous vertical stirring for 16 minutes. The mixture was left in a dark place at laboratory temperature for 9-10 hours. The extract was filtered using a filter device with a pore size of 0.45 µm, connected to a medical syringe. Hexane (99%) was added in a volume of 1 ml to concentrate the extract and remove water. The upper liquid was then withdrawn from the filtration solution. The extract is now ready to estimate the active chemical compounds in the aquatic *Azolla* plant using gas chromatography-mass spectrometry (GC-MS).

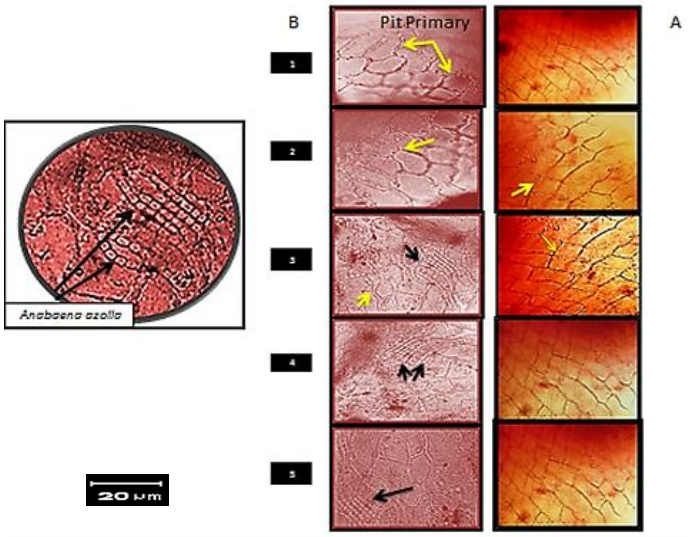

Figure 1. Variations in the shapes of epidermal cells of *Azolla pinnata* treated with light intensity (LED) at different levels. (A) represents the upper epidermis and B the lower epidermis, (⇦) the primary pit fields, and (→) *Anabaena azolla.* 1: Control, 2: 15000 lux light intensity, 3: 10000 lux light intensity, 4: 5000 lux light intensity, and 5. 2000 lux light intensity treatments.

Results and Discussions

Effect of different light intensities on the anatomical characteristics of *A. pinnata* leaves.

***Epidermis*:** The results showed that differences in light intensity affected the anatomical and chemical characteristics of the leaves of *A. pinnata*. The epidermal cells were almost regular and tended to be rectangular on the upper surface, and the cells on the lower surface were similar in shape to those of the upper epidermis, in addition to having a sub-semi-circular polygonal shape. The walls in both epidermises were straight to curved. Primary pit fields were evident on both surfaces, especially on the lower surface, which was marked by the presence of *Anabaena azolla* algae (Fig. 1). The upper and lower epidermal surfaces were characterized by the presence of cells consisting of three to four or more transparent rows called hyaline cells (Fig. 2). These results align with what Kostka et al. (2016) stated, that these cells

Table 1. Variations in the quantitative characteristics of the upper and lower surfaces of the epidermis of *Azolla pinnata* leaves treated with light intensities (LED) at different levels (measured in micrometers).

	Upper epidermis		Lower epidermis	
The plant species	Ordinary epidermal cell dimensions		Ordinary epidermal cell dimensions	
A. pinnata	**length**	**Width**	**length**	**Width**
control	5-8 (6.66)	3-5 (4.16)	5-7 (6.22)	3.5-6 (4.85)
15000 lux	5.5-8 (6.75)	4.5-6 (4.55)	5.3-7.5 (6.88)	3.5-7 (5.85)
10000 lux	5.5-7 (6.75)	3-5 (4.17)	5-8 (6.83)	3.5-8 (5.85)
5000 lux	5-6.5 (5.66)	3-5 (4.17)	5-6.5 (5.83)	3.5-4.5 (4.08)
2000 lux	5-6 (5.06)	3-4.5 (4.16)	5-5.5 (5.03)	3.5-5 (4.14)

The numbers outside the parentheses represent the upper and lower limits. The numbers inside the parentheses represent the average.

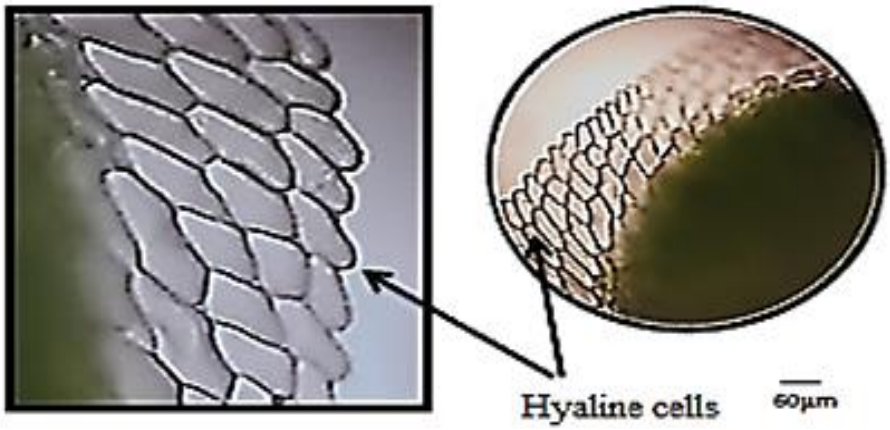

Figure 2. Hyaline cells in the upper and lower epidermis of *Azolla pinnata*.

are characterized by their lack of chlorophyll pigments and that their function is to store and exchange water between them and the adjacent epidermal cells. One distinguishing characteristic of the epidermis of this plant is the absence of stomata on the upper and lower surfaces. The presence of these cells also agreed with the findings of Alkhafaje (2021) when studying the species *A. filiculoides*, but did not agree regarding the presence of stomata, as the current study showed the absence of stomata on both surfaces of the *Azolla* plant. Ali (2022) pointed out that some aquatic plants are characterized by the absence of stomata, allowing gases to be exchanged through their cell walls.

Anabaena Azolla algae was also observed, with its density increasing at light intensities of 5,000 and 2,000 lux. The study's results confirmed, as El-Hawary and El-Kholy (2019) indicated, the importance of *Anabaena Azolla* in *Azolla* plants. This represents a symbiotic relationship that links the algae and the plant, enhancing the plant's ability to tolerate environmental factors such as light, nutrients, and other conditions.

The dimensions of *A. pinnata* epidermal cells treated with different light intensities overlapped, with the highest rate for the upper surface ranging between 6.75x4.55 µm when the plant was treated with light intensities of 15,000 and 10,000 lux. The lowest rate was observed in 2,000 lux, reaching 5.06x4.16 µm. For the lower surface, the highest rate ranged from 6.88x5.85 µm at a light intensity of 15,000 lux. The lowest rate of epidermal cell dimensions was 5.03x4.14 µm at a light intensity of 2,000 lux compared to the control treatment (Table 1). This variation may be attributed to differences in light intensity, which led to changes in the thickness and shape of the plant cell walls, as well as differences in the dimensions of the epidermal cells on both the upper and lower surfaces of the plant. Bibi et al. (2023) stated that one reason for the differences in epidermal cell walls is the adaptation of aquatic plants to varying environmental conditions, prompting them to respond by altering the thickness of their cell walls.

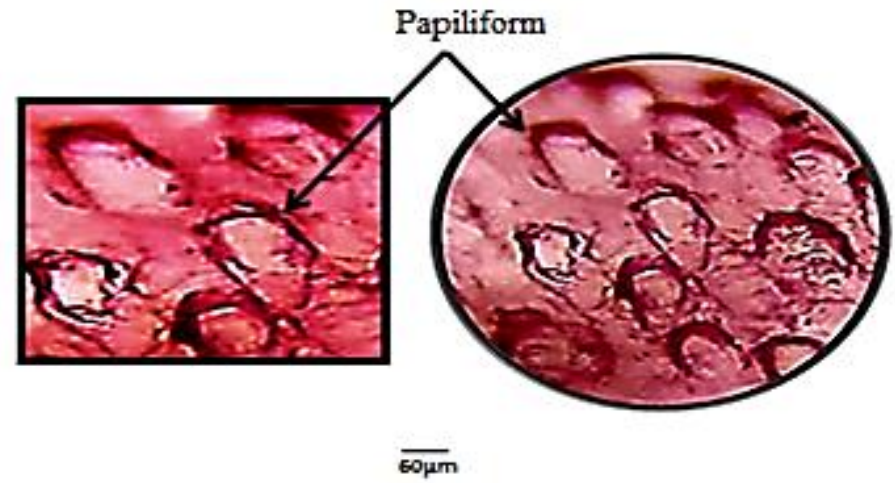

Figure 3. Papiliform papillae in the upper epidermis of *Azolla pinnata* in the control treatment.

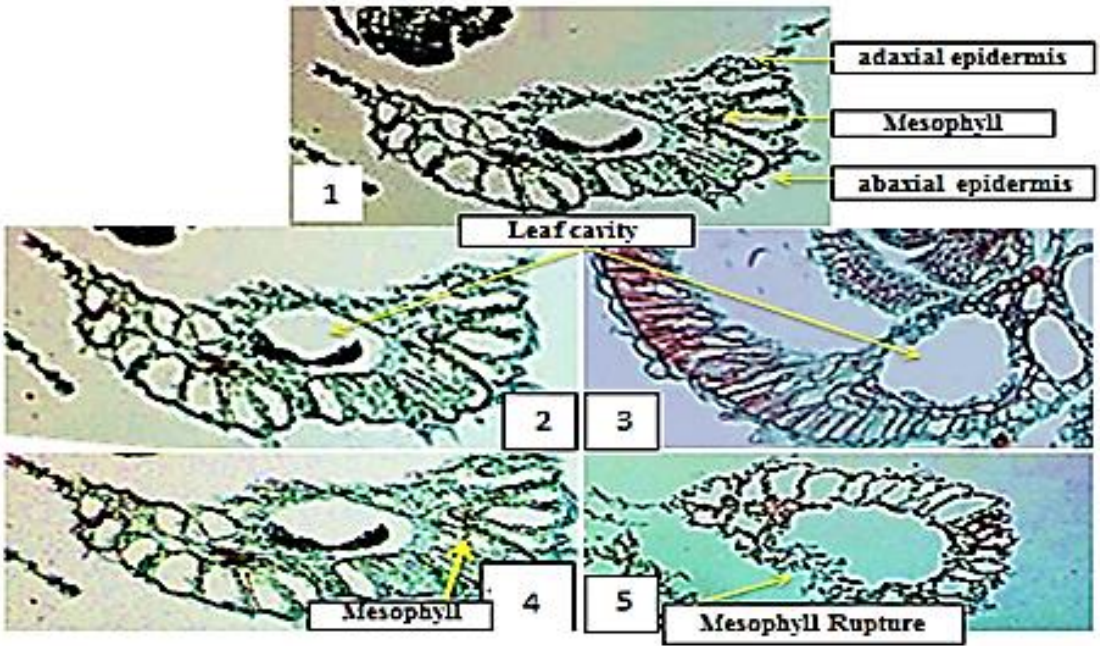

Figure 4. Cross-sections of *Azolla pinnata* leaves treated with different light intensity (LED) (scale bar 20 µm). 1. Control treatment plants, 2. Plants treated with 15000 lux light intensity, 3. Plants treated with 10000 lux light intensity, 4. Plants treated with 5000 lux light intensity, 5. Plants treated with 2000 lux light intensity.

The results were consistent with those of Washeeh et al. (2024) when they studied the *E. crassipes*, confirming the effect of different light intensities (LED) on the epidermal anticlinal cell walls and their thickness, as well as on the dimensions of the epidermal cells.

The plant indumentum is represented by the presence of oval papillae with a rough texture on the upper surface of the epidermis in the control treatment only (Fig. 3). This finding aligns with Al-Khafaji (2021), who noted the presence of such papillae in the epidermis of other *Azolla* species. The study also agrees with Salih (2023) and Washeeh et al. (2024) that plant indumentum is affected by the environmental factors to which the plant is exposed. Our results confirmed this, as such papillae disappeared when exposed to different light intensities compared to the control treatment.

Transverse sections of *A. pinnata* leaves: The results of the present study on the cross-sections of *A. pinnata* leaves treated with light intensity (LED) at different levels showed that the plant leaf consists of two lobes:

Table 2. Variations in quantitative characteristics of cross-sections of *Azolla pinnata* leaves treated with light intensities (LED) at different levels (measured in micrometers).

Treatments	Cuticle thickness	Epidermis thickness		Mesophyll tissue thickness
		Upper epidermis	Lower epidermis	
control	4.1-1.3 (3.2)	19.2-15.8 (17.3)	24.1-17.3 (23.5)	36.2-28.8 (33.5)
15.000 lux	4.4-1.2 (3.3)	16.3-11.1 (14.5)	19.2-12.6 (17.5)	42.1-38.2 (41.5)
10.000 lux	4.4-1.5 (3.5)	18.6-23.5 (22.1)	28.1-19.6 (27.5)	46.3-40.5 (44.2)
5.000 lux	3.4-2.5 (3.2)	25.5-17.1 (20.5)	18.6-20.6 (27.5)	41.5-36.1 (41.6)
2.000 lux	3.4-1.1 (2.3)	14.1-18.2 (13.6)	20.7-26.2 (25.4)	37.2-33.5 (36.5)

The numbers outside the parentheses represent the upper and lower limits. The numbers inside the parentheses represent the average.

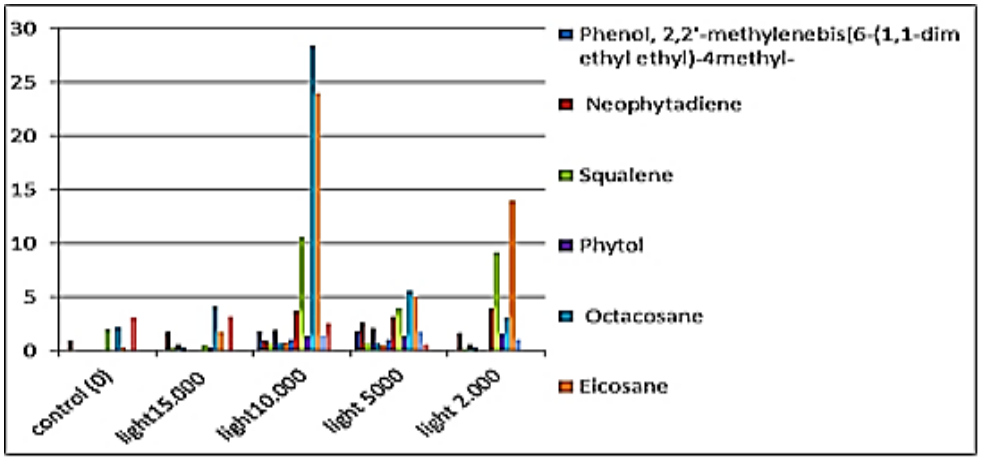

Figure 5. GC-MS analysis of the active compounds in *Azolla pinnata* under different light intensities.

the upper or dorsal lobes located in the upper epidermis, and the ventral lobe in the lower epidermis. The *Azolla* leaf also contains a leaf cavity in the ventral lobe (Fig. 4). The mesophyll tissue consists of two layers: a spongy tissue layer and a palisade tissue layer, which have columnar shapes and vary in size from one treatment to another and may differ within a single plant treatment. The epidermal cells were distinguished by the absence of stomata in *A. pinnata*.

The highest average thickness of the mesophyll layer (44.2 µm) was recorded when the plant was treated under a light intensity of 10,000 lux, and the lowest average thickness (36.5 µm) was recorded in a low light intensity of 2,000 lux compared to the control treatment. Additionally, the *A. pinnata* epidermis was characterized by being surrounded by a continuous cuticle layer that varied in thickness; the highest thickness reached 3.5 µm when the plant was treated with a light intensity of 10,000 lux, while the lowest thickness (2.3 µm) was recorded in the 2,000-lux treatment (Table 2).

The difference in light intensity resulted in variations in the thickness of the epidermis in *Azolla* plants. As the light intensity increased to 10,000 lux, the thickness increased, while a sharp decrease in light intensity led to a reduction in the thickness of the epidermis to 13.6 µm on the upper surface. For the lower surface, light intensities of 10,000 and 5,000 lux resulted in increased thickness, whereas high light intensity (15,000 lux) decreased thickness compared to the control treatment (Table 2). This may be attributed to the sharp decrease and significant increase in illumination affecting the thickness of the epidermal tissue and differences in the dimensions of the mesophyll tissue, as tearing was observed when the light intensity sharply decreased to 2,000 lux (Fig. 4). These results were consistent with the findings of Al-Saadi et al. (2000) and Al-Kulaby (2015), who stated that differences in environmental conditions lead to variations in most anatomical characteristics in plants, such as the thickness of the mesophyll tissue and epidermal layers, as well as the air spaces. This was confirmed in the current study, as the increase in light led to an expansion of the air spaces, likely due to increased gas exchange to reduce photo-oxidative stress and cool the plant cells. Houry et al. (2021) and

Table 3. The results of the analysis (GC-MS) of the active compounds in the leaves of the plant *Azolla pinnata* under the influence of different levels of LED lighting intensity ((-) = absence).

compound type	sequencing	chemical composition	Formula Chemical	Name of compound	plant type \ *A.pinnata* Compound concentration = (area %) \ R. Time									
					Light 0		Light 115.000 lux		Light10.000 lux 2		Light5.000 lux 3		Light 42.000 lux	
					concentration	RTime	concentration	RTime	concentration	RTime	concentration	RTime	concentration	RTime
Phenolic compounds	1		C10H10O4	Vanillin, acetate	-	-	0.38	6.62	-	-	-	-	-	-
	2		C23H32O2	Phenol, 2,2'-methylenebis [6-(1,1-dim ethyl ethyl)-4methyl-	-	-	-	-	1.86	15.95	1.82	15.96	-	-
Terpenes Compounds	3		C20H38	Neophytadiene	0.95	11.12	1.74	11.13	0.94	11.54	2.64	11.12	1.60	11.13
	4		C30H50	Squalene	-	-	0.17	19.38	0.69	19.39	0.66	19.40	0.15	19.38
	5		C20H40O	Phytol	-	-	0.59	13.16	1.89	13.63	2.06	13.62	0.61	13.35
alkanes	6		C18H32O7	Butyl citrate	2.91	13.71	-	-	-	-	-	-	5.56	14.07
	7		C28H58	Octacosane	-	-	0.21	18.46	0.63	16.18	0.62	16.18	0.19	18.18
	8		C20H42	Eicosane	-	-	-	-	0.73	10.76	0.50	12.15	-	-
	9		C16H34	Hexadecane	-	-	-	-	1.05	9.75	1.02	9.77	-	-
	10		C40H82O2	Hexadecane, 1,1-bis (dodecyloxy)-	0.43	11.38	-	-	-	-	1.02	13.32	-	-
Ami A	11		C19H38O2	Methyl stearate	-	-	-	-	3.66	13.71	3.16	13.71	3.96	13.71
Fatty acid	12		C17H34O2	Hexadecanoic acid, methylester	1.99	11.82	0.45	12.48	10.60	11.83	3.97	11.83	9.10	11.83
	13		C22H42O2	Erucic acid	-	-	0.21	18.82	1.37	18.81	1.41	18.84	1.52	18.82
	14		C19H36O2	10-Octadecenic acid, methylester	2.15	13.45	4.14	13.50	28.40	13.45	5.60	13.45	3.11	13.51
	15		C23H44O2	13-Docosenoic acid,methylester (Z)-	0.28	16.88	1.72	16.84	23.95	16.85	5.00	16.85	13.95	16.85
	16		C16H31NO	Palmitoleamide	-	-	0.51	15.51	-	-	-	-	-	-
	17		C7H12O5	Glycerol 1, 2-diacetate	-	-	0.49	5.53	-	-	-	-	-	-
	18		C18H34O2	Oleic acid	-	-	-	-	1.33	13.77	1.72	13.77	0.99	13.35
carbohydrates	19		C21H15F12NO9	L(-)-Fucose, tetrakis(trifluoroacetat), benzyloxime (isomer 2	3.09	3.28	3.12	3.27	2.50	3.28	0.59	3.19	-	-

Al-Khatari (2023) noted that most plants are affected by various factors, especially light, which is reflected in some anatomical characteristics, such as the thickness of the cuticle layer surrounding the plants and differences in the epidermis and plant tissues as adaptations to protect the plant. This may be due to the increased activity of antioxidant enzymes that stimulate the plant to add materials to the surface and walls of plant cells, resulting in variations in wall thickness. Barros et al. (2015) and Balk et al. (2023) agreed that antioxidant enzymes, such as catalase (CAT), peroxidase (POD), and superoxide dismutase (SOD), increase in activity when the plant experiences stress from different environmental factors, including light. These enzymes participate in various physiological reactions, such as cell growth and development, and stimulate the lignification process, which involves adding materials to plant cell walls, contributing to the plant's adaptation to surrounding environmental factors and increasing thickness. From a chemical perspective, lignin is considered a polyphenolic compound produced during these enzymes' oxidation of free radicals.

Effect of different light intensities on the chemical compounds of *A. pinnata* leaves: The results of the current research study showed that the analysis of chemical compounds for plant extracts from the leaves of *A. pinnata*, under the influence of varying light intensities revealed the presence and disappearance of certain chemical compounds in some plant treatments. A total of nineteen diverse compounds were recorded, including phenols, terpenes, alkanes, amino acids, fatty acids, and carbohydrate compounds. The light intensity of 15,000 lux led to the appearance of the phenolic compound vanillin acetate, and light intensities of 10,000 and 5,000 lux resulted in the appearance of the phenolic compound phenol, 2,2'-methylenebis[6-(1,1-dimethyl ethyl)-4-methyl-] (Table 3, Fig. 5). The two terpene compounds, squalene, and phytol, were also observed at all light intensity levels, which were not present in the control treatment.

The difference in light intensity also affected the appearance and disappearance of some alkane compounds, in addition to the varying concentrations of all compounds among the different light treatments compared to the control treatment (Table 3, Fig. 5). Regarding amino and fatty acid compounds, their presence and absence varied compared to the control treatment. The amino acid methyl stearate appeared in all treatments but was absent in the control treatment. Seven fatty acid compounds were identified, differing in their appearance and disappearance and the concentration of each compound according to the light intensity (Table 3, Fig. 5). Low light intensity (2,000 lux) led to the disappearance of the carbohydrate compound L(-)-fucose, tetrakis (trifluoroacetate), and benzyl oxime (isomer 2) compared to the control treatment.

The appearance and disappearance of chemical compounds may be attributed to the plant's response to differences in light intensity, which helps protect its cells from damage caused by increased photo-oxidative stress, which may rise with fluctuations in light intensity. This variation in intensity can affect the biochemical activities within the plant, prompting it to convert some chemical compounds into others, leading to the appearance or disappearance of certain compounds. The results align with the findings of Ma et al. (2010), who stated that these changes are primarily due to physiological alterations occurring within the plant. The study also corroborates the work of several researchers regarding the significant impact of light on chemical compounds in plants when using different levels of LED, such as Hasan et al. (2017), Ye et al. (2017), and Jung et al. (2021). This was further confirmed by the study of Santin et al. (2021) on the effect of lighting technology (LED) on active compounds in *E. crassipes*, an aquatic plant. They noted that variations in light affect the chemical and physiological reactions within the plant, leading to the conversion of various antioxidants, such as terpene compounds, which increase the secondary pigment beta-carotene, as well as phenols that form the antioxidant pigment anthocyanin, in addition to amino and fatty acids that contribute to an increase in enzymatic antioxidants. Washeeh et al. (2024) showed that on *E. crassipes* light significantly influences the

conversion of carbohydrates into fatty acids in more significant proportions than amino acids. Therefore, there is a greater opportunity for the formation of oils compared to proteins, which was confirmed by the current study. It was noted that there was a lack of carbohydrates in the plant under study, and some carbohydrates were absent in other treatments.

Conclusion

It can be concluded that different light intensities led to variations in the anatomical properties of the cross-sections of the leaves and their epidermis, as well as differences in the surface cover. For example, higher light intensity resulted in thicker epidermal and mesophyll tissues. One distinguishing characteristic of the epidermis of this plant is the absence of stomata on both the upper and lower surfaces. The study showed that different light intensity levels significantly affected chemical compounds, causing certain compounds' appearance and disappearance, e.g., the phenolic compound vanillin acetate appeared when the light intensity increased significantly to 15,000 lux, and the alkane compound butyl citrate disappeared under higher light conditions.

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