Long-term and short-term responses of morphology, anatomy, stomatal and photosynthesis of *Arachis pintoi* to light stress

Nguyen Tien Anh Huy and Do Thuong Kiet*
Department of Plant Physiology, Faculty of Biology and Biotechnology, University of Science, VNU-HCM, Ho Chi Minh City, VIETNAM
*dtkiet@hcmus.edu.vn

Abstract

Pinto Peanut (Arachis pintoi Krapov. and W.C. Greg) is beneficial for reducing greenhouse gas emissions, enhancing nitrogen fixation and serving as an important biomass for livestock and medicinal purposes. As a leguminous plant, Pinto Peanut prefers humid and shaded conditions, forming overlapping canopies but also shows long-term adaptation to excessive light conditions. In this study, the changes in morphology, anatomical structure and physiology of Arachis pintoi leaves were analyzed after long-term excess light stress. Additionally, leaf damage and recovery after short-term light stress were also examined.

The results showed that new growth shoots formed under direct sunlight had smaller sizes, narrower leaf areas and doubled stomatal density compared to the control. Although strong light reduced chlorophyll fluorescence density in the leaves, starch presence remained high. For leaves subjected to short-term treatment, morphological damage was mainly concentrated in the spongy mesophyll. This indicates that increased transpiration and the sacrifice of spongy mesophyll are adaptive strategies of the leaves. While morphological damage and reduced stomatal aperture could not be recovered, overnight recovery of pigments and gas exchange activity of photosynthesis were observed.

Keywords: *Arachis pintoi*, excess light, chlorophyll fluorescent, stomata, photoinhibition, photodamage, photosynthesis recovery.

Introduction

Pinto Peanut (*Arachis pintoi* Krapov and W. C. Gregory) is a perennial creeping legume. The growth of this plant can form dense swards, making it a potential forage crop and a source of important secondary compounds for medicine. This plant thrives in shaded conditions and high soil moisture but can also tolerate excessive light exposure and dehydration in open areas. Legumes have high water usage, suitable for regions with humidity above 75%. Soil moisture below 50% begins cause to stress the plant, rapidly damaging the leaves, which have numerous stomata[8]. Leguminous plants have expanded stomata, enhanced transpiration and facilitated CO_2 uptake for photosynthesis and biomass accumulation. This is their main strategy for adapting to different moisture levels[9].

Leguminous plants, such as soybeans, have stomatal density directly affected by drought stress during leaf growth and development. Their leaves significantly reduced stomatal density on the leaf surface in drought stress[6]. Besides drought stress, light stress also has a strong impact and often accompanies or causes localized drought stress in leaves. Excessive light affects gas exchange, energy conversion and energy balance in photosynthesis, thereby controlling biomass accumulation. High light intensity (over 20 klux) reduces plant height and leaf area but increases dry matter accumulation in stems and roots[11]. Excessive light damages the photosynthetic apparatus of plants and algae primarily through reactive oxygen species (ROS)[5]. ROS formed in the photosystem II reaction center causes photoinhibition[10]. Photodamage, caused by ROS, leads to reduce the content of photosynthetic pigments related to the light-harvesting complex (LHCII)[5]. In this study, we investigate the morphological, structural and physiological changes in the leaves of Pinto Peanut under long-term excessive light treatment and recovery after short-term treatment.

Material and Methods

Plant materials and growth conditions: Shoot cuttings with four leaves of Pinto peanut (*Arachis pintoi* Krapov. and W.C. Greg) were collected under shade (control) at the University of Science, Linh Trung Ward, Thu Duc City, Ho Chi Minh City, Viet Nam. The intact leaflets of the fifth leaves were collected after two-week-old plants in the shade.

Long-term excess light treatment: Shoot cuttings with four leaves were collected from the Pinto peanut (*Arachis pintoi* Krapov. and W.C. Greg) under shade. The stems were soaked with a little distilled water in test tubes in the plant growth room with light intensity at 3000 lux, temperature at 33 ± 1ºC, humidity at 60 to 65% and the 12/12 photoperiod for two days and after being transferred to soil pots (8 x 8 x 8 cm) in the experimental garden under shade and direct sunlight for two weeks. Plant shape, anatomy structure, stained anatomy structure, chlorophyll fluorescent, leaf area, thickness, fresh weight, gas exchange and stomata aperture of the leaflet of the fifth leaves were recorded after two weeks of plant growth.

Short-term excess light treatment and recovery analysis: Plants from shoot cutting under shade in the experimental garden were transferred to the growth room, illuminated with light intensity at 3k lux, temperature at 33 ± 1ºC,

humidity at 60 to 65% and the 12/12 photoperiod. Then, the intact leaflets of the fifth leaves were directly illuminated with light intensity at 80k lux for 8 hours a day from 10:00 to 18:00. The stomatal aperture, gas exchange and pigments content of the leaflets were recorded in the following chronological timeline: (A) before light treatment at 10:00 AM, (B) after 8 hours with the light treatment at 18:00 and (C) 16 hours after the light treatment at 10:00 AM.

Observation of leaf anatomy and starch-stained anatomical structure: The intact leaflets were detached, soaked in little distilled water and cut across the secondary vein of the leaf. The horizontal slices were observed directly or stained with Lugol solution and then observed under the microscope. The leaflet anatomy of the fifth leaves was observed under a microscope. In addition, the leaflet anatomy was also stained with Lugol solution. Anatomical and fluorescent images were recorded with a Leica camera by LAS EZ Imaging software.

Evaluation of fluorescence excitation screening: The horizontal slices of secondary veins of leaves were observed under the fluorescent microscope (Olympus CKX41) with the excitation wavelength at 488 nm and filter at 560 nm. Chlorophyll fluorescent levels of leaf anatomy were analyzed with ImageJ software. RGB fluorescent images were split into red, green and blue channels. Fluorescent regions were chosen with a threshold to get fluorescent levels. Fluorescent levels range from 0 to 255, with corresponding red color values from 0 to 255.

Determination of chlorophyll content index: Leaflets were clamped at the sensor of the SPAD-502 chlorophyll meter (Konica Minolta Sensing, Inc., Sakai, Osaka, Japan). The SPAD value shows the ratio transmission of light between 650 nm and 940 nm wavelength [7]. The chlorophyll content index (SPAD) was the average of four values from four leaflets on the leaf.

Stomatal aperture measurement: The stomatal shape was fixed by cyanoacrylate on microscope slides [12]. The stomata images were captured and compared with a micrometric slide (Optika, Italia, M-005) at the same degree of magnification. Then, the stomatal apertures were measured with ImageJ software.

Gas exchange measurement: Plants were transferred to darkness for 5 minutes. Next, the leaflets of the fifth leaves were detached and put in the Gas-Phase Oxygen Electrode Chambers (LD2/3 Electrode Chamber, Hansatech Instruments) under light intensity at 3000 ± 500 lux, the temperature was maintained at 28.0ºC ± 0.2ºC by a cooling system. Then, the gas exchange of leaflets was recorded for 5 minutes and the values were recorded from the second to the fifth minute with O_2View Software.

Determination of chlorophylls and carotenoids content: *Arachis pintoi* leaves were ground with acetone 100% at 4ºC in darkness. The extracts were centrifuged at 6000 rpm for 5 minutes to separate supernatants and the process was repeated three times. The supernatants were collected and diluted with acetone 100% to 10 mL. The absorbance of the supernatants was recorded at 470 nm, 645 nm and 662 nm wavelength with the visible spectrophotometer (GENESYS™ 30 Visible Spectrophotometer, Thermo Scientific™). The pigments content (chlorophyll a, b and carotenoids) and ratio of chlorophylls were determined[4].

Results and Discussion

Responses of Arachis pintoi plant and leaves under long-term excess light: Shoot growth of *Arachis pintoi* plants from shoot cuttings was reduced after two weeks in excess light, particularly in direct sunlight. In recent studies, the growth of *Arachis pintoi* was better under shade [3]. However, this plant could adapt and tolerate high light intensity during the day. In a long-term excess light for two weeks, physiological changes would occur in leaves, especially the diminution of leaf area [8]. The area of the fifth of leaves was higher under shade than under direct sunlight.

Furthermore, water storage tissues of this plant are concentrated on the lower leaf epidermis and larger than under shade. This could help this plant to maintain osmotic pressure in leaves to resist high light intensity in direct sunlight. This means that water is necessary for the growth and development of *Arachis pintoi* in direct sunlight. At the same time in two conditions at 10:00 AM, chloroplasts of the plant under shade were widely distributed throughout mesophyll cells including palisade and spongy mesophyll cells. Meanwhile, chloroplasts were arranged parallel in palisade mesophyll cells and were concentrated in spongy mesophyll cells.

The leaflets area was higher in shade than in sunlight. This showed that the growth of *Arachis pintoi* was inhibited in sunlight. Compared to shade, the part of the palisade mesophylls was destroyed in direct sunlight light. This led to the decrease of chlorophylls in palisade mesophylls. Therefore, the chlorophyll fluorescent level was lower in direct sunlight than in shade. With the leaflet anatomy structure stained with Lugol solution, the starch was concentrated in palisade mesophyll under direct sunlight while wider distributed in mesophylls under shade, including palisade and spongy mesophyll.

This demonstrated that *Arachis pintoi* photosynthesis belongs to C3 plants. In the previous studies, the growth of C3 plants was less efficient in hot and dry conditions and was adapted to a broader range of environmental conditions. In the leaflet anatomy, large spongy mesophyll cells were concentrated on the lower epidermis and these cells had an important role in storing water. This could help this plant to tolerate high light intensity in direct sunlight. The spongy mesophylls size was larger in shade than in direct sunlight. Moreover, the thickly waxy cuticle of leaflets prevented transpiration from the leaf surface and the process had

mainly occurred through stomata. This showed that the cuticle on the lower epidermis was still shaped in direct sunlight, even though the area of the large spongy mesophylls was decreased by water lose.

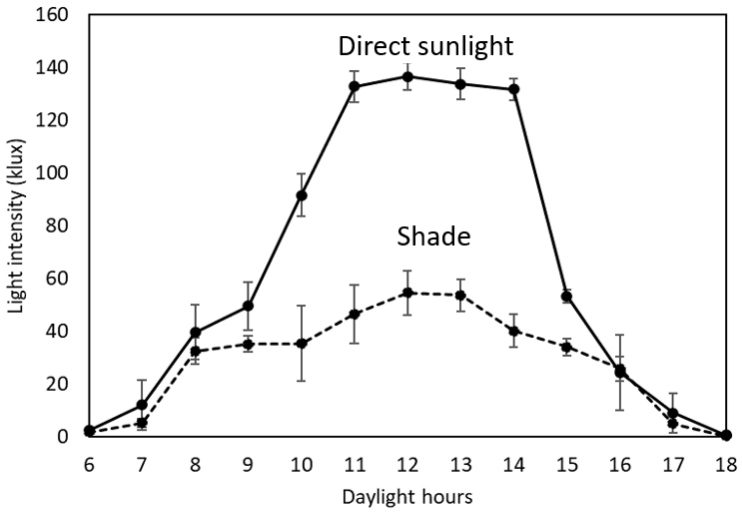

Figure 1: Light intensities during the day in shade and direct sunlight.

Figure 2: *Arachis pintoi* was planted from the shoot cuttings after two weeks (A) under shade and (B) full light, (C) the leaflets of a fifth leaves under shade and (D) direct sunlight.

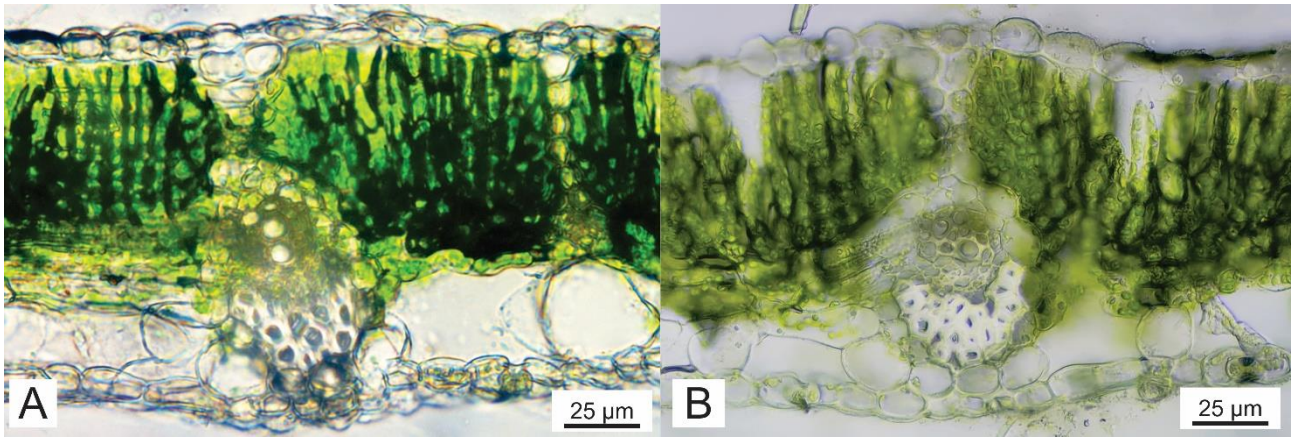

Figure 3: Leaflet anatomical structure from the fifth leaves (A) under shade and (B) direct sunlight.

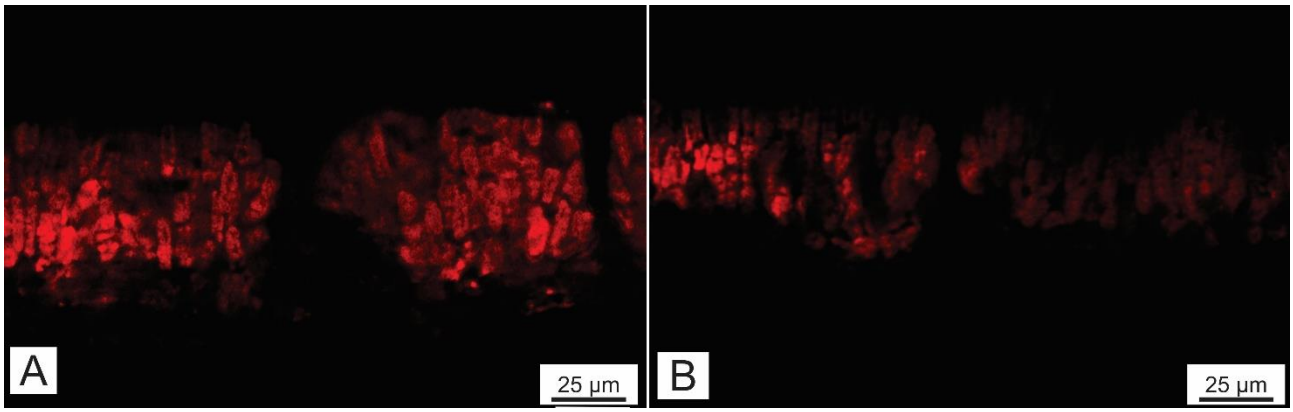

Figure 4: The chlorophyll fluorescence of leaflet anatomical structures of the fifth leaves (A) under shade and (B) direct sunlight was observed with a fluorescence microscope, using the excitation wavelength at 488 nm and the filter at 560 nm.

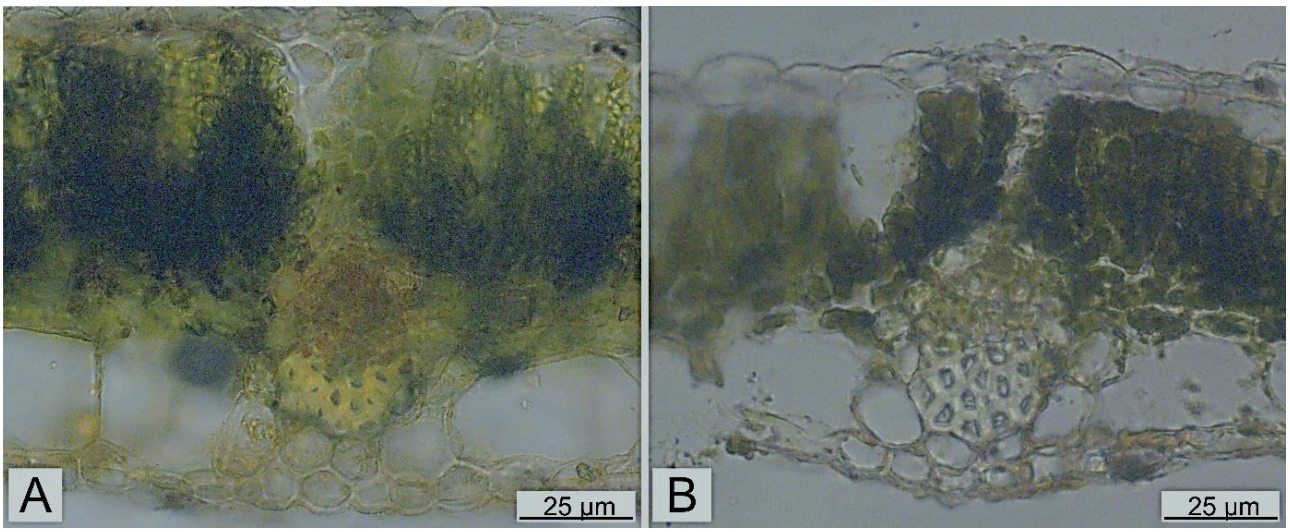

Figure 5: Leaflet anatomical structures of the fifth leaves (A) under shade and (B) direct sunlight stained with Lugol solution

Chlorophyll fluorescent, starch accumulation and photosynthesis activities of the leaves significantly changed under shade and direct sunlight. Compared to shade, leaf area, chlorophyll-a content and gas exchange were decreased under direct sunlight. The results showed that the high light intensity in direct sunlight decreased leaf photosynthesis activities, due to reduced growth of top shoot cuttings after two weeks. Moreover, the photosynthesis activities were higher in shadethan in direct sunlight. As a result, energy from photosynthesis was used for plant growth in shade. This energy was used to recover injured cells, caused by the high light intensity in direct sunlight. This led to reduced plant growth in direct sunlight.

Besides, some changes in leaf anatomy structure such as chlorophyll fluorescent and spongy mesophyll, different physiological changes help this plant adapt to the excess light intensity of sunlight such as reducing leaf area, thickness and fresh weight. Increasing water loss in the spongy mesophyll could help the plants to adapt to excess light. Moreover, the light stage photosynthetic was assessed based on chlorophyll fluorescence, chlorophyll index and pigment content in leaves. As a result, the leaf photosynthesis was reduced in excess light after two weeks, showing a decreasing chlorophyll content index (SPAD) and chlorophyll a content in leaves.

However, there were no differences in protective pigments, including chlorophyll b and carotenoids after two weeks between shade and direct sunlight. In the same leaf area, reducing leaf size was associated with increasing stomatal density in leaves. The number of stomata in leaves had no decrease under excess light stress, unlike under drought stress[9]. Compared to shade, gas exchange is lower in direct sunlight than shade. This demonstrated that excess light decreased gas exchange, reduced photosynthesis productivity and inhibited plant growth after two weeks.

Leaf tolerance evaluation in short-term light stress and recovery capability after light stress: The intact leaflet of *Arachis pintoi* was strongly damaged under excess light treatment for 8 hours from 10:00 to 18:00. The colors of the leaflets were changed from green to lighter green and the brown region appeared in the cuticle on the upper leaf epidermis after excess light treatment. This showed that leaf pigment content could be decreased under excess light, mainly chlorophyll a content and carotenoids in palisade mesophylls. In particular, the chlorophyll fluorescent was reduced almost completely under excess light. To adapt to excess light, water loss occurred in spongy mesophylls, decreased leaf thickness, fresh weight and reduced stomatal opening on the lower leaves. Therefore, gas exchange and photosynthesis of leaves were decreased after excess light. So, the growth of plants and leaves could be inhibited in the short-term excess light treatment.

Especially, the damages caused by short-term excess light treatment could partially recover overnight. Particularly, chlorophyll fluorescent of palisade mesophylls recovered 30% after excess light. This indicates that the productivity of photosynthesis could be recovered, leading to improved oxygenic photosynthesis in leaves[1]. Moreover, the chlorophyll a content insignificantly increased, but the chlorophyll a and b ratio increased after excess light treatment. This means that oxygenic photosynthesis could be recovered by increasing chlorophyll activity, especially chlorophyll a in photosystem II.

Table 1
Leaf area, leaflet area, leaf thickness and fresh weight under shade and direct sunlight.

Light conditions	The fifth leaf area (cm^2)	Leaflet area (cm^2)	Leaflet thickness (µm)	Leaflet fresh weight (mg)
Shade	9,96 ± 0,72	2,78 ± 0,32	109.9 ± 2.5	130 ± 8
Direct sunlight	7,53 ± 0,51	1,65 ± 0,12	88.7 ± 2.8	113 ± 5
t-Test	+	+	+	+

* Results showed the difference between shade and direct sunlight at $p \leq 0.05$ with the independent samples t-test and presented as mean ± standard deviation.

Table 2
Chlorophyll content index, pigments content of leaflets from the fifth leaves under shade and direct sunlight.

Light conditions	Chlorophyll content index (SPAD)	Pigments content (µg/mm^2)			a/b ratio
		Chl a	Chl b	Carotenoids	
Shade	42.27 ± 1.76	0,38 ± 0,11	0,05 ± 0,02	0,08 ± 0,02	7,84 ± 3,61
Direct sunlight	40.41 ± 1.30	0,23 ± 0,05	0,07 ± 0,02	0,06 ± 0,01	3,36 ± 1,71
T-test	+	+	–	–	–

* Results showed the difference between shade and direct sunlight at $p \leq 0.05$ with the independent samples t-test and presented as mean ± standard deviation.

Table 3
Stomatal density, number of stomata and gas exchange of leaflets on the fifth leaves in shade and sunlight.

Light conditions	Stomata density (No. Stomata/mm^2)	No. Stomata/ leaflet	Gas exchange (µmol O_2/m^2/s)
Shade	417 ± 60	168 ± 21	22,43 ± 1,61
Direct sunlight	845 ± 48	185 ± 10	14,76 ± 2,47
t-Test	+	–	+

* Results showed the difference between shade and direct sunlight at $p \leq 0.05$ with the independent samples t-test and presented as mean ± standard deviation.

Besides, the palisade mesophyll structure seems to be unaffected after excess light treatment. This demonstrated that the excess light treatment with light intensity at 80 klux for 8 hours a day had caused leaf photodamage, could recover photosynthesis overnight and could not cause the death of leaves. In addition, water loss in spongy mesophylls was not recovered overnight. There were no significant changes in leaf fresh weight and thickness after excess light treatment. Therefore, the stomatal opening was not recovered after excess light. This demonstrated that excess light increased stomatal closure in *Arachis pintoi* leaves. The increasing stomatal closure could not completely inhibit the gas exchange after excess light. This was a change to help provide CO_2 for photosynthesis and to recover photosynthesis activities after excess light[2].

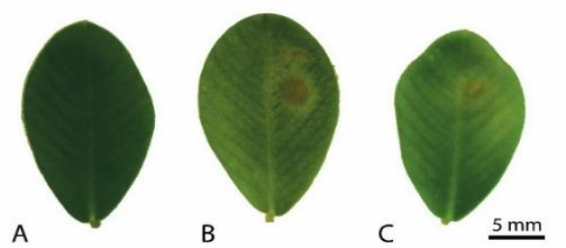

Figure 6: The intact leaflets were illuminated with excess light intensity at 80 klux at the plant growth room in the following chronological timeline: T0, before excess light treatment with 80 klux at 10:00 AM (A); T1, 8 hours after light stress illuminating with 80 klux at 6:00 PM (B); T2, 16 hours after light stress illuminating in light intensity at 3000 lux at 10:00 AM (C).

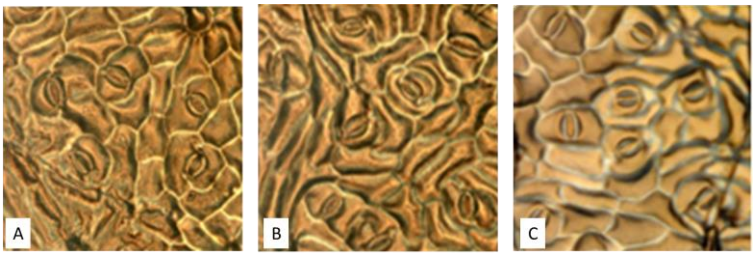

Figure 7: Stomatal opening of the leaflets of the fifth leaves after excess light treatment in the following chronological timeline: (A) T0, before excess light treatment with 80 klux at 10:00 AM; (B) T1, 8 hours after light stress illuminating with 80 klux at 6:00 PM; (C) T2, 16 hours after light stress illuminating in light intensity at 3000 lux at 10:00 AM.

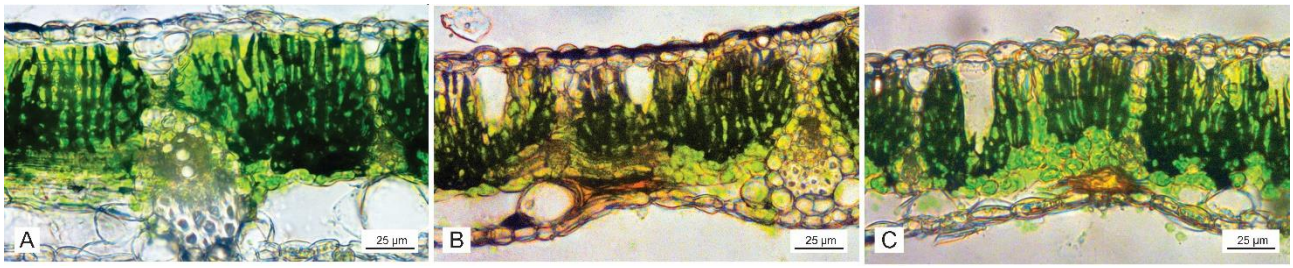

Figure 8: Leaflet anatomical structure of the fifth leaves after excess light treatment in the following chronological timeline: (A) T0, before excess light treatment with 80 klux at 10:00 AM; (B) T1, 8 hours after light stress illuminating with 80 klux at 6:00 PM; (C) T2, 16 hours after light stress illuminating in light intensity at 3000 lux at 10:00 AM.

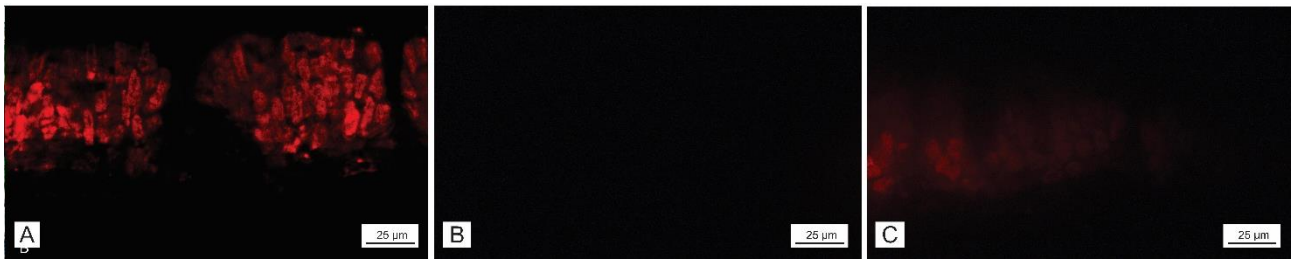

Figure 9: Chlorophyll fluorescence of leaflet anatomical structure of the fifth leaves under (A) a light intensity of 3 klux, (B) 8 h of light treatment at 80 klux, (C) 16 h after the end of light treatment at 80 klux was observed under fluorescent microscope at excitation wavelength 480 nm and the filter at 560 nm

Table 4
Stomata aperture and photosynthesis intensity of leaflet on the fifth leaves after light stress for 8 hours.

Excess light treatment Timeline *	Fresh weight (mg)	Leaflet thickness (µm)	Stomata aperture (µm)	Gas exchange (µmol $O_2/m^2/s$)
T0	45.66 ± 6.62 [a]	128.5 ± 3.1 [a]	3,52 ± 0,08 [a]	27,44 ± 1,48 [a]
T1	30.22 ± 9.70 [b]	119.5 ± 4.4 [b]	2,32 ± 0,14 [b]	2,22 ± 0,86 [c]
T2	35.94 ± 6.62 [b]	113.9 ± 6.9 [b]	2,38 ± 0,09 [b]	11,11 ± 1,91 [b]

* T0, before excess light treatment with 80 klux at 10:00 AM (A); T1, 8 hours after light stress illuminating with 80 klux at 6:00 PM (B); T2, 16 hours after light stress illuminating in light intensity at 3000 lux at 10:00 AM (C).

Table 5
Chlorophyll fluorescent index of the leaflets on the fifth leaves after light stress for 8 hours.

Excess light treatment Timeline	Chlorophyll fluorescent	Pigment content (µg/mm^2)			
		Chlorophyll a	Chlorophyll b	Carotenoids	Chl a/b ratio
T0	131.6 ± 12.6 [a]	0,32 ± 0,01 [a]	0,10 ± 0,01	0,08 ± 0,00 [a]	3,13 ± 0,22 [a]
T1	2.0 ± 0.7 [c]	0,19 ± 0,03 [b]	0,13 ± 0,02	0,04 ± 0,01 [b]	1,41 ± 0,12 [c]
T2	38.4 ± 12.9 [b]	0,26 ± 0,03 [ab]	0,11 ± 0,01	0,06 ± 0,01 [a]	2,28 ± 0,10 [b]

* T0, before excess light treatment with 80 klux at 10:00 AM (A); T1, 8 hours after light stress illuminating with 80 klux at 6:00 PM (B); T2, 16 hours after light stress illuminating in light intensity at 3000 lux at 10:00 AM (C).

Conclusion

New growth shoots formed under direct sunlight had smaller sizes, narrower leaf areas and doubled stomatal density compared to the control (shade). Long-term excess light for two weeks under direct sunlight decreased chlorophyll a content, prevented chlorophyll fluorescent in palisade mesophylls, starch presence remained high and increased lost water in spongy mesophylls.

Short-term excess light caused damage to leaves concentrated in the spongy mesophyll. Increasing transpiration and water loss in spongy mesophyll are adaptive strategies of the leaves. Morphological damage and reduced stomatal aperture could not be recovered. Overnight recovery of pigments and gas exchange activity of photosynthesis were observed.

Acknowledgement

This research was funded by the University of Science, VNU-HCM, under grant number T2024-43. The experiments were conducted at the Department of Plant Physiology, Faculty of Biology and Biotechnology, University of Science, VNU-HCM, 72711 Ho Chi Minh City, Viet Nam.

References

1. Baker N.R. and Oxborough K., Chlorophyll fluorescence as a probe of photosynthetic productivity, In Chlorophyll a fluorescence: a signature of photosynthesis, Springer, 65-82 **(2004)**

2. Ferroni L., Brestič M., Živčak M., Cantelli R., Pancaldi S.J.P.P., Increased photosynthesis from a deep-shade to high-light regime occurs by enhanced CO2 diffusion into the leaf of Selaginella martensii, *Plant Physiol Biochem*, **160**, 143-154 **(2021)**

3. Fisher M. and Cruz P., In Kerridge P.C. and Hardy B., eds., Biology and agronomy of forage Arachis, Centro Internacional de Agricultura Tropical (CIAT), 53-70 **(1994)**

4. Lichtenthaler H.K. and Wellburn A.R., Determinations of total carotenoids and chlorophylls a and b of leaf extracts in different solvents, Portland Press Ltd., 591-592 **(1983)**

5. Lingvay M., Akhtar P., Sebők-Nagy K., Páli T. and Lambrev P.H.J.F., Photobleaching of chlorophyll in light-harvesting complex II increases in lipid environment, *Frontiers in Plant Science*, **11**, 849 **(2020)**

6. Mangena P., Water stress: morphological and anatomical changes in soybean (Glycine max L.) plants, Plant, abiotic stress and responses to climate change, 9-31 **(2018)**

7. Nauš J., Prokopová J., Řebíček J. and Špundová M., SPAD chlorophyll meter reading can be pronouncedly affected by chloroplast movement, *Photosynthesis Research*, **105**, 265-271 **(2010)**

8. Sales R.M.P. et al, Effect of water stress on the growth of Arachis pintoi plants under different nitrogen levels, *Revista Brasileira de Zootecnia*, **42**, 149-154 **(2013)**

9. Singh S.K. and Reddy K.R., Regulation of photosynthesis, fluorescence, stomatal conductance and water-use efficiency of cowpea (Vigna unguiculata [L.] Walp.) under drought, *Journal of Photochemistry*, **105(1)**, 40-50 **(2011)**

10. Sun Z. et al, Resilient and sustainable production of peanut (Arachis hypogaea) in phosphorus-limited environment by using exogenous gamma-aminobutyric acid to sustain photosynthesis, *Ecotoxicology Environmental Safety*, **263**, 115388 **(2023)**

11. Tang W. et al, Effect of light intensity on morphology, photosynthesis and carbon metabolism of alfalfa (Medicago sativa) seedlings, *Plants*, **11(13)**, 1688 **(2022)**

12. Wilson C.K.L., Pusey P.L. and Otto B.E., Plant epidermal sections and imprints using cyanoacrylate adhesives, *Canadian Journal of Plant Science*, **61(3)**, 781-783 **(1981)**.

(Received 21th October 2024, accepted 23rd November 2024)