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Nagamatsu López, Yuriko; Blanco Labra, Alejandro; Délano Frier, John; Pimienta Barrios, Eulogio
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INTENSIDAD LUMINOSA Y ACTIVIDAD DE INHIBIDORES DE TRIPSINA EN HOJAS Y SEMILLAS DE AMARANTO

LIGHT INTENSITY AND ACTIVITY OF TRYPSIN INHIBITORS IN AMARANTH LEAVES AND SEEDS

Yuriko Nagamatsu López[1], Alejandro Blanco Labra[2], John Délano-Frier[2] y Eulogio Pimienta Barrios[1*]

[1] Departamento de Ecología, Centro Universitario de Ciencias Biológicas y Agropecuarias, Universidad de Guadalajara. Km 15.5 Carretera a Nogales. C. P. 45110 Las Agujas, Zapopan, Jalisco, México. Tel. y Fax: 01 (333) 777-1156. Correo electrónico: epimient@cucba.udg.mx [2]Departamento de Biotecnología y Bioquímica, Centro de Investigaciones y Estudios Avanzados del Instituto Politécnico Nacional. Unidad Irapuato. Km 9.6 Libramiento Carretera Irapuato-León. C. P. 36500 Irapuato, Guanajuato, México. Tel. 01 (462) 623-9640
** Author for correspondence*

SUMMARY

The effect of exposing *Amaranthus hypochondriacus* plants to different levels of photosynthetic photon flux densities (PPFD) during development was analyzed in this work. Mature plants exposed to full sunlight (38.8 mol m^{-2} d^{-1}) had higher instantaneous rates of net CO_2 uptake (A_n), reducing sugars content and thicker leaves, but lower chlorophyll content and leaf number per plant than those developed under reduced levels of PPFD (19.4 and 12.8 mol m^{-2} d^{-1}). A physiological response to varying levels of PPFD was the differential accumulation of trypsin inhibitors in leaves and seeds. Leaves from plants grown under full sunlight conditions, showed a significantly higher trypsin inhibitor activity than leaves from plants partially shaded with plastic nets. In contrast, seeds collected from plants fully exposed to sunlight, showed the lowest level of trypsin inhibitors and higher rates of germination than seeds produced by plants exposed to the lowest level of sunlight. The capacity of *A. hypochondriacus* to adjust its morphology and physiology in response to light indicates ecological plasticity that might be helpful to face both biotic and abiotic stresses during their development.

Index words: *Amaranthus hypochondriacus* Mill., flujo fotosintético de fotones, inhibidores de tripsina, intercambio de gases, clorofila, anatomía foliar, germinación de semillas.

RESUMEN

En este trabajo se estudió el efecto de la exposición de plantas de *Amaranthus hypochondriacus* a diferentes niveles de densidad de flujo fotosintético de fotones (PPFD) durante su desarrollo. Las hojas de plantas maduras expuestas a luz solar plena (38.8 mol m^{-2} d^{-1}) presentaron tasas instantáneas de asimilación de CO_2 (A_n) más altas, mayor contenido de azúcares reductores y hojas más gruesas, pero menor contenido de clorofila y menor número de hojas por planta, que las plantas que crecieron bajo niveles reducidos de PPFD (19.4 y 12.8 mol m^{-2} d^{-1}). Una respuesta fisiológica a la variación en los niveles de PPFD fue la acumulación diferencial de inhibidores de tripsina en hojas y semillas. Las hojas de plantas que crecieron bajo condiciones de luz solar plena, mostraron un incremento significativo en la actividad de inhibidores de tripsina en las hojas, en comparación con hojas de plantas parcialmente sombreadas. En contraste, las semillas de las plantas expuestas a la luz solar total, mostraron niveles más bajos de inhibidores de tripsina, y tasas más altas de germinación que las semillas producidas en plantas expuestas a los niveles menores de radiación solar. La capacidad de las plantas de *A. hypochondriacus* para ajustar su respuesta morfológica y fisiológica a la luz, es una indicación de plasticidad ecológica que puede ser de utilidad para enfrentar estreses bióticos y abióticos durante su desarrollo.

Palabras clave: *Amaranthus hypochondriacus* Mill., photosynthetic photon flux, trypsin inhibitors, gas exchange, chlorophyll, leaf anatomy, seed germination.

INTRODUCTION

Protease inhibitor proteins (PIs) have a defensive role in plants against chewing insects and certain pathogens (Lorito *et al*., 1994; Koiwa *et al*.,1997). A typical response to mechanical wounding or insect attack is the induced accumulation of a characteristic family of trypsin and chymotrypsin-PIs in tomato (*Lycopersicum esculentum* Mill.) and potato (*Solanum tuberosum L.*) plants. In these species, PIs accumulate both locally in wounded leaves and systemically in the peripheral, unwounded leaves (Ryan, 1990). Various PIs are expressed in a constitutive manner, whereas others accumulate in an organ-specific fashion after induction by biotic or abiotic elicitors or concomitantly with plant growth and development (Botella *et al*., 1996).

Amaranthus hypochondriacus Mill. is a C_4 pseudocereal that has the potential to be cultivated successfully due to its ability to produce reasonable yields in infertile soils and semiarid conditions, mostly characterized by high light

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intensities and high diurnal temperatures. It is also remarkably tolerant to insect pests and its seeds have proteins with a high nutritive value (Kauffman and Weber, 1990). Because little information is available on the abiotic factors, particularly light, that may affect PIs synthesis, an experiment was performed to elucidate the possible connection existing between light, photosynthesis and the accumulation of trypsin inhibitors in leaves and seeds of *A. hypochondriacus*. For this purpose, three groups of plants were exposed to different light intensities and their effect on photosynthesis and trypsin inhibitor production was evaluated. The results obtained indicate that light is an important factor affecting differentially the synthesis and accumulation of trypsin inhibitors in both vegetative and reproductive tissues of this plant species.

MATERIALS AND METHODS

Plants growth conditions

This study was conducted from February to September 2000, at the Experimental Research Station of the Departamento de Ecología of the Universidad de Guadalajara, Jalisco, México. The site is located in the central region of the state of Jalisco, México, at 20° 05' NL, 103° 32' WL, and 1420 m above sea level. Climate is classified as temperate-subtropical.

Seeds of *Amaranthus hypochondriacus* Mill. cv. Nutrisol, were collected from one progenitor in the third week of February and were immediately planted in 48 plastic containers. Ten days later, each seedling was assigned to one of the three light treatments replicated 16 times, in a completely randomized design. One group was covered with a plastic net that attenuated 67 % of the incident sunlight (equivalent to a total irradiance of 12.8 mol m^{-2} d^{-1} ± 71.51); in the second group, also covered with a plastic net, incident sunlight was attenuated 50 % (19.4 mol m^{-2} d^{-1} ± 108.35), and the third group was exposed to full sunlight (38.8 mol m^{-2} d^{-1} ± 216.69).

The average photosynthetic photon flux density (PPFD) for all treatments, were calculated on the basis of the seven independent measurements made during the course of the experiment on March 17, April 4, May 26, May 31, June 8, July 12 and August 2. In every determination, PPFD was measured on a horizontal plane using a Li-Cor 190S quantum sensor, at 1-h intervals from 8 to 20 h. The instantaneous values recorded were integrated in order to determine the total daily PPFD. Twenty-four leaf sections, two from each of twelve plants for each light treatment, at 11 weeks after emergence, were taken with a cork-borer (1.54 cm of diameter). The leaf sections were used for the biochemical and physiological determinations. At this point, plants were fully mature and ready to enter the flowering stage.

Plant characteristics, seed weight and seed germination

Leaf number per plant was determined 105 days after sowing. On 10 plants per treatment, leaf thickness was measured with a Zeiss microscope at 125× magnification on 10 leaf transverse sections per plant. Seed weight was recorded by measuring the combined weight of 320 seeds (20 seeds per plant) taken from each treatment. Germination efficiency was tested on six replicates of 20 seeds placed on two layers of Whatman No. 1 filter paper at 20 °C under constant light (355 μmol m^{-2} s^{-1}) in a 9-cm-diameter Petri dish, and soaked with 8 mL of distilled water. Germination was scored when the radical length surpassed 2 mm, and expressed as the percentage of germinated seeds within 10, 20 and 30 days of imbibition treatment.

Gas exchange measurements

Instantaneous rates of net CO_2 uptake (A_n) and stomatal conductance (g_s) were measured on twelve plants per treatment, on June 8, 2000, 105 days after sowing. Measurements were performed at two-hour intervals from 8:00 to 18:00, using a Li-Cor LI-6200 portable photosynthesis system (Li-Cor, Lincoln, NE, USA) equipped with a 250 cm^3 Li-Cor leaf chamber. Air temperature was also recorded every hour with a mercury thermometer.

Soluble sugars and chlorophyll content

Ten leaf sections (1.54 cm^2) of each of twelve plants per treatment were cut off from leaves on the same dates of gas exchange measurements using a cork-borer and kept frozen until analyzed. Soluble reducing sugars were extracted from leaves by homogenizing 1 g of frozen tissue in a blender with 5 mL of aqueous ethanol (80 %) at 75 °C for 5 min. The extract was centrifuged at 1400 *g* for 5 min and the supernatant was recovered. The pellet was reextracted and centrifuged as before. The supernatants were combined and centrifuged at 22 000 *g* at 4°C for 30 min in a centrifuge Beckman J2-21 (Carnal and Black, 1989). Aliquots were taken to measure the reducing sugar content, expressed as glucose equivalents, according to the colorimetric method described by Somogyi (1952). Chlorophyll content was determined in extracts prepared from frozen 2-g leaf samples of the same plants. Extractions were performed by rapidly homogenizing leaf material in cold acetone (80 %). The homogenates were centrifuged for 10 min at 12 000 *g* at 4 °C. The insoluble plant material was re-extracted and centrifuged, as above. Both

supernatants were combined and centrifuged one more time, under the same conditions, to eliminate fine particles. Care was taken to avoid light exposure, by covering the extracts with aluminum foil. Chlorophyll content (μg mL^{-1}) was determined on the basis of the light absorbed at 663 nm and after 645 nm in the same extract (Bruinsma, 1961).

Trypsin-inhibitory activity

Leaf sections (1.54 cm^2) were cut from the leaves as described in the previous section, frozen in liquid nitrogen, and ground into fine powder in a mortar. The ground material was suspended in 600 μL of deionized-distilled water and subsequently homogenized for 5 min. The mixture was centrifuged at 17 000 *g* for 15 min at 4 °C. The supernatant was re-centrifuged at the same speed for 1 min to eliminate the remaining plant debris. The resulting extracts were assayed for protein content using the Bradford method (Bradford, 1976). The extracts produced numerous protein bands capable of inhibiting bovine trypsin when tested in reversed gel zymograms (Délano-Frier, pers. comm.)[1].

Inhibitory activity against bovine trypsin was determined as described by Schwertz and Takenaka (1955) and expressed as trypsin inhibitor units (TIU) per mg of protein. This method quantifies inhibition on the basis of changes in the rate of hydrolysis of benzoyl arginine ethyl ester (BAEE) by bovine trypsin, which can be followed at 254 nm. BAEE is a substrate specifically recognized by trypsin and trypsin-like enzymes. Protein content and trypsin-inhibitory activity in seeds were determined as above, using aqueous extracts prepared from 0.05 g of ground seed flour.

Statistical analysis

Data were subjected to analysis of variance and when significant, means were separated by the least significant difference (LSD) test.

RESULTS

Both average leaf number and leaf chlorophyll content increased in response to a reduction in light availability. Thus, in mature *A. hypochondriacus* plants, leaf number increased more than triple, as light intensity decreased 33 % of total sunlight (Table 1). In contrast, leaf thickness, reducing sugar content, and the specific activity of trypsin inhibitors increased with light availability, being significantly higher in fully exposed plants (Table 1).

A_n values for *A. hypochondriacus* plants increased sharply in the morning hours, along with an increase in PPFD in plants fully exposed to sunlight and in plants that received only 50 % of the total solar radiation. Plants of *A. hypochondriacus* that grew under 33 % total solar radiation, showed a notorious weaker response to light during the morning (Figures 1A, 1C). The highest A_n values were recorded at noon in fully exposed plants and those exposed to 50 % of the total solar radiation, whereas for plants exposed to 33 % solar the highest A_n values were recorded until late afternoon. Plants exposed to full sunlight and 50 % of the total solar radiation showed A_n depression after noon (Figure 1A). Diurnal net CO_2 uptake obtained by integrating the instantaneous rates over a 10-h period, was approximately 317 mmol m^{-2} d^{-1} for plants fully exposed to sunlight, and 209 and 112 mmol m^{-2} d^{-1} for shaded plants grown under 50 and 33 % of the total incident sunlight, respectively (Table 1).

Regardless of light exposure, values of g_s of *A. hypochondriacus* increased during the morning (8 to 12 h) (Figure 1B). Unexpectedly, g_s values were highest in plants kept under 33 % of the solar radiation. Stomatal conductance decreased just past noon (Figure 1B), a response that coincided both with high levels of PPFD (Figure 1C), and with air temperatures above 30 °C (Figure 1D).

Seeds collected from plants exposed to full sunlight were lighter and contained lower trypsin inhibitor levels than seeds from shaded plants. They also showed a higher percentage of germination during the first 20 days after water imbibition than seeds collected from shaded plants (Table 2). Trypsin inhibitor activity was the highest in those seeds produced by plants kept under the lowest light intensity. During the seed germination process, 80 % of seeds from fully exposed plants were contaminated with *Aspergillus niger*, in contrast with seeds of plants exposed to 50 and 33 % of sunlight, which showed only 50 and 10 % of infection, respectively.

DISCUSSION

Amaranthus hypochondriacus showed an array of morphological and physiological adaptations in response to different light intensities. The lowest number of leaves recorded in fully exposed plants is the result of a high level of leaf abscission occurring in fully illuminated *A. hypochondriacus* plants. Commonly, leaf abscission is an avoidance mechanism to decrease water loss in response to an increased evaporative demand (Evans and Black, 1993). However, in these experiments plants experienced marked

[1] John Délano-Frier. Bioquímica vegetal. Centro de Investigación y de Estudios Avanzados-Instituto Politécnico Nacional, Unidad Irapuato.

Table 1. Morphological and physiological responses of Amaranthus hypochondriacus *leaves growing under three PPFD conditions, measured at 105 days after sowing.*

Sunlight exposure (%)	Total daily photosynthetic photon flux (mol m^{-2} d^{-1}) ±s.e.	Leaf number per plant	Leaf thickness (μm)	Diurnal net CO_2 uptake (mmol m^{-2} d^{-1}) (A_n)	Leaf chlorophyll (μg g^{-1})	Leaf reducing sugars (mg g^{-1})	Leaf trypsin inhibitor activity (TIU mg^{-1})
100	38.8± 216.69	35a	322 a	317.6	69 b	0.521 a	45 a
50	19.4± 108.35	77a	273 b	209.2	109 a	0.419 ab	26 b
33	12.8± 71.51	111a	229 c	112.5	143 a	0.389 b	20 c

Data are means, except for net CO_2 uptake (n = 16 for leaf number, 100 for leaf thickness, 12 for leaf chlorophyll, 12 for leaf reducing sugars and 12 for trypsin inhibitor activity). Mean values within a column followed by the same letter are not statistically different at $P<0.02$ by LSD's multiple test. S.E.= Standard error; TIU= Trypsin inhibitor units.

Table 2. Weight, trypsin inhibitor specific activity, and germination rates from seeds produced by Amaranthus hypochondriacus *growing under three PPFD conditions.*

Sunlight exposure (%)	Total daily photosynthetic photon flux (mol m^{-2} d^{-1}) ± s.e.	Seed weight (μg)	Seed trypsin inhibitor activity (TIU mg^{-1})	Germination rate (%)		
				10 days	20 days	30 days
100	38.8± 216.69	593 b	17 b	65 a	82 a	87 a
50	19.4± 108.35	678 a	22 ab	52 ab	70 ab	84 a
33	12.8± 71.51	687 a	27 a	24 b	40 b	82 a

Data are means (n = 320 for seed weight, 12 for trypsin inhibitor activity and 6 for seed germination rate). Mean values within a column that are followed by the same letter are not statistically different at $P<0.05$ by LSD's multiple test. s.e.= Standard error; TIU= Trypsin inhibitor units.

differences in PPFD but not in air temperatures, to account for differences in rates of transpiration or water loss between shaded and fully exposed plants.

Leaves have the capacity to structurally and physiologically acclimate to differences in light availability. Plants of *A. hypochondriacus* that received one-third of full sunlight, showed morphological and physiological characteristics similar to those of shaded plants, typically producing thinner leaves with high chlorophyll content (Lambers *et al.*, 1998). The observed reduction in leaf chlorophyll of plants exposed to full sunlight is thought to be a mechanism to reduce photo-induced damage (Horton, 2000). On June 8, for example, irradiation levels, which varied from 1600 to 2100 μmol m^{-2} s^{-1}, were usually measured from 11 h to 15 h at the study site. These values have been considered to cause light-related damage due to bulk pigment loss (Long *et al.*, 1994). Thus, the observed reduction in leaf area under full sunlight conditions might be part of a strategy to reduce photodamage in the photosynthetic tissue, without affecting the light trapping efficiency.

C_4 plants like *A. hypochondriacus* have higher photosynthetic rates than C_3 and CAM plants (Nobel, 1999), particularly when they grow in open habitats in which high temperatures and high light incidence prevail (Sage, 2001). Although plants of *A. hypochondriacus* were exposed to high levels of PPFD and warm temperatures, the maximum rate of A_n (16 µmol m^{-2} s^{-1}) was comparatively small in comparison to values recorded for other C_4 plants, which often show maximum A_n values from 25 to 60 µmol $m^{-2}s^{-1}$ (Nobel, 1999). Even relatives of *A. hypochondriacus*, as is the case of *A. palmeri* and *A. edulis* (Smith *et al.*, 1997; Bailey *et al.*, 2000), show rates of net CO_2 uptake that varied from 20 to 60 µmol m^{-2} s^{-1}. Old leaves of *A. edulis* assimilated CO_2 more slowly than those recently expanded; perhaps leaf age could be a determinant factor causing the low A_n values measured in *A. hypochondriacus* plants (El-Sharkawy *et al.*, 1968).

Plants of *A. hypochondriacus* exposed to full-sunlight and to 50 % total solar radiation, had rates of CO_2 uptake that showed a linear response to light early morning (8 to 12 h); then, both A_n and g_s decreased just past noon. During midday, net photosynthesis inhibition is commonly attributed to high temperatures (Cowan, 1995; Matos *et al.*, 1998), drought (Larcher, 1995), high PPFD levels (Marler *et al.*, 1994; Pathre *et al.*, 1998), and to an increase in vapor pressure deficit (Mohotti and Lawlor, 2002) that causes stomatal closure (Sinclair and Allen, 1982), and photoinhibitory damage (Mohotti and Lawlor, 2002).

The afternoon decrease in A_n and g_s could be attributed to partial stomatal closure caused by high temperatures and high levels of PPFD (Marler *et al.*, 1994), that regularly induced temporal water stress (Cowan, 1995; Pathre *et al.*, 1998). However, this inhibition of

photosynthesis and stomatal closure by water stress is considered to be merely coincidental, since many studies on gas exchange revealed that the relationship between g_s and A_n is weak (Kozlowski *et al*., 1991; Pereira, 1995; Long, 1999). A more plausible scenario is that the reduction in A_n might be related to non-stomatal factors such as the inhibitory effects of high PPFD concomitant to high air temperatures, causing damage to the PSII reaction center (Mohotti and Lawlor, 2002) and reducing quantum yield (Marler *et al*., 1994).

Leaves of *A. hypochondriacus* plants exposed to direct sunlight showed both the highest rates of carbon assimilation and highest levels of reducing sugars. These results suggest a possible positive correlation between soluble sugar concentration and trypsin inhibitor levels.

Protease inhibitors are associated with plant defense responses against wounding or herbivory insects (Ryan, 1990). Therefore, the presence of a light-related accumulation of trypsin inhibitors in leaves of *A. hypochondriacus* could have been part of a constitutive defense mechanism that requires, as in tomato plants (Ryan, 1990), high levels of PPFD to be efficiently expressed. Trypsin inhibitor accumulation in leaves of *A. hypochondriacus* plants exposed to full sunlight could have been, in addition to increased leaf thickness, part of a protective mechanism against damaging ultraviolet (UV) light levels present in intense solar radiation (Conconi *et al*., 1996).

In contrast to its effect on leaves, light intensity was inversely correlated with size and trypsin inhibitor accumulation in seeds. Thus, seeds collected from *A. hypochondriacus* plants exposed to full sunlight were the smallest, had the lowest levels of trypsin inhibitor activity and showed the fastest rates of germination. Our results also suggest a connection between low levels of trypsin inhibitor activity in seeds of *A. hypochondriacus* and high rates of germination, because the trypsin-like proteolytic activity required to release the nutrient reserves in the endosperm utilized during the germination process, is less attenuated.

Trypsin and chymotrypsin inhibitors have been shown to inhibit fungal and fungal proteases growth (Lorito *et al*., 1994). It could be argued then that the lower levels of trypsin inhibitor activity detected in seeds derived from fully illuminated *A. hypochondriacus* plants could have been responsible for the higher susceptibility to fungal colonization observed during germination. However, more experimentation is required to establish a firm relationship between trypsin inhibitors in seeds of *A. hypochondriacus* and protection against fungal invasion.

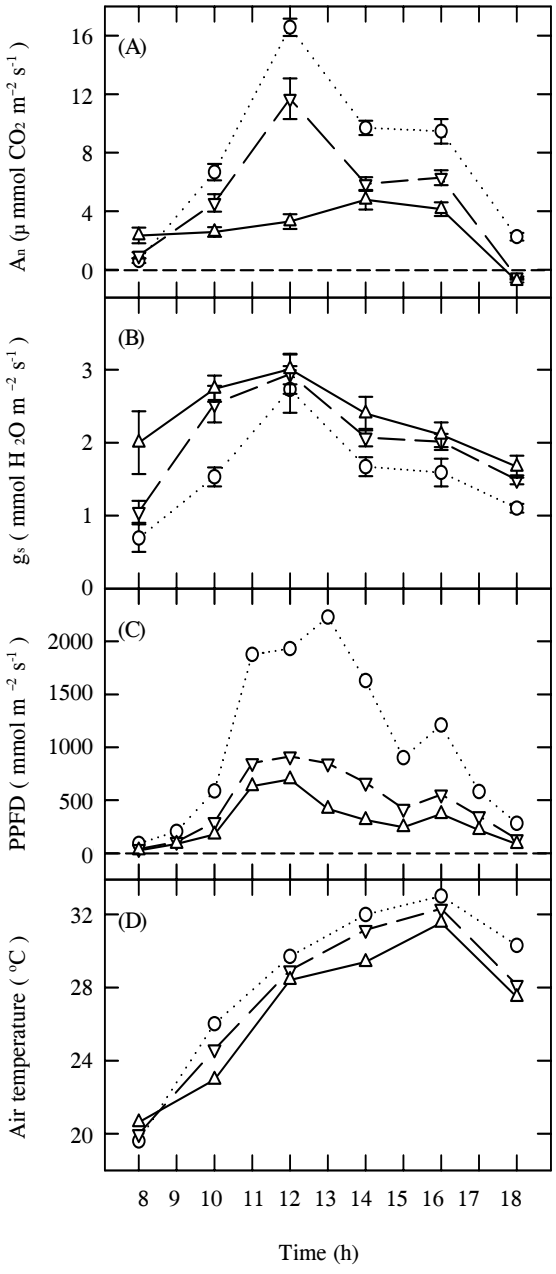

Figure 1. (A) Net CO_2 uptake (A_n) and (B) stomatal conductance (g_s) for leaves of Amaranthus hypochondriacus plants exposed to full sunlight (··O··), or shaded plants covered with nets that intercepted 50% (-- ▽--), and 67% (▵▵) of solar radiation. (C) Photosynthetic photon flux density (PPFD) on a horizontal plane and (D) air temperature under each light regime. Data were collected from measurements made on June 8, 2000 at Guadalajara, Jalisco, México. Graphs (A) and (B) show mean values and standard errors of 12 measurements.

CONCLUSIONS

Amaranthus hypochondriacus showed physiological plasticity to both high and low levels of light modulating its reaction through morphological and physiological responses.

Light intensity was found to have a differential effect on trypsin inhibitor levels in *A. hypochondriacus* plants. The effect was tissue specific, since it promoted the accumulation of trypsin inhibitor activity in leaves and repressed it in seeds. The direct relationship observed between trypsin inhibitor activity and reducing sugar levels in leaves suggests that light regulation of trypsin inhibitor activity is dependent on the concentration of soluble sugars produced during active photosynthesis. The increased levels of foliar trypsin inhibitor activity in plants exposed to full sunlight, suggest that trypsin inhibitors in *A. hypochondriacus* could have a protective role against stress caused by high light and temperature.

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