

Diurnal photoperiods and rhythmicity of the phototropic bending response in hypocotyls of sunflower, *Helianthus annuus* L. seedlings

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Abstract: Research on phototropic (PT) bending in sunflower (*Helianthus annuus* L. cv. Kondi (Syngenta)) seedling hypocotyls presented herein focused on a comparison of diurnal and free-running photoperiods with the aim of explaining the development of diurnal rhythmicity. PT bending magnitudes and lag phase duration exhibited strong daily rhythmicity in all diurnal photoperiods, contrasting with the uniform PT bending response to constant light (CL) conditions. Plants had a daytime maximum for PT bending magnitudes in experiments starting around midday and a minimum in the dark period in those starting 4 h after dusk. Plants could compensate for large differences in the daytime duration of diurnal photoperiods. They required the first 4 h of darkness to recover and synchronize the PT bending and to start increasing the magnitudes of PT bending. The daily pattern of lag phase duration changes was similar but inverted, showing that synchronization also occurred during nighttime. Darkness was not required for PT bending under CL conditions, however, during diurnal photoperiods it enabled the establishment of diurnal rhythmicity and synchronized changes in PT bending capacity to occur when needed, providing maximal values at midday and minimal during the nighttime. Under prolonged duration of daytime corresponding to the start of CL condition, plantlets rapidly abandoned circadian regulation, their PT bending response becoming arrhythmic.

Keywords: phototropism; sunflower; photoperiods; diurnal rhythmicity; circadian regulation

INTRODUCTION

Phototropism is a growth response triggered by incoming light that enables plants to adjust the spatial position of their shoot and root systems. Shoot phototropism of dicotyledonous plants was traditionally studied in species of horticultural interest like *Lepidium* [1], *Pisum* [2], *Fagopyrum* [3] and others, until *Arabidopsis* in the mid-1980s became the main model system for phototropism with initial techniques described in [4]. One of the many advantages of the *Arabidopsis* model system was that it enabled studies of both first and second positive phototropism [5]; however, work in *Arabidopsis* and other species was temporarily focused on the more interesting first positive responses of etiolated seedlings.

In *Arabidopsis*, phototropism is caused by differences in the cell elongation rates on the irradiated and shaded shoot flanks [6]. The main pigments of

phototropism are phototropins [7], although their action can be supported and extended by other blue- and red-light absorbing pigments such as phytochromes and cryptochromes [8,9].

The blue light signal perceived by appropriate pigments is transferred by a complex network of recipients and events, arranged in a number of distinct steps [10] until it reaches the effectors driving the phototropic (PT) bending responses. Turgor and hydraulic signaling associated with the movement of water across the shoot are possible effector candidates both for phototropism and circumnutations as indicated [11-14].

In sunflower seedlings, vigorous PT movements are first exhibited in hypocotyls, seedling shoots that are active only for the first 6-7 days after germination. In this short time, plantlets complete the development of cotyledons, establish photo-autotrophic metabolism and then gradually transfer PT bending capacity to the

internodes developing above the cotyledons. In sunflower plants, the hypocotyl provides strong vascular bundles for cotyledons [11] and functions as a junction connecting the root and shoot vascular systems.

The gravitropic (GT) bending response of sunflower seedling hypocotyls is much stronger than their PT bending responses [15]. For this reason, it is difficult to establish and maintain photogravitropic equilibrium as the one used for quantitative study of tropistic responses in coleoptiles of oats [16].

The phototropic movements of sunflower hypocotyls have been thoroughly studied [15,17-21], but there have been no recent updates nor new studies in the last four decades. Almost all previous studies were performed during the 14/10 h light:darkness diurnal photoperiod, which is similar to the conditions under which plants are raised for commercial field production.

PT bending of sunflower hypocotyls towards a unilaterally positioned light source follows a ≈ 3 -h-long rhythm imposed by interaction with gravitropism, which starts later but then overtakes the PT bending [15]. For this reason, PT bending has a conspicuous maximum followed by a decrease in the PT bending angle (straightening). Phototropic bending is absent in dark-grown, etiolated sunflower seedlings [15,17] in which it appears only upon de-etiolation. Thus, phototropism can be studied only in light-grown sunflower plants and seedlings, as in the case of potato shoots [22]. Blue light is the only component of the visible light spectrum that can trigger and drive phototropic bending, while red light can only modify the blue light-induced PT bending response [15].

Sunflower seedlings are also known for their vigorous circumnutations, autonomous circular-like movements [13,23] favored in the absence of light [24]. They were used as test objects in some NASA space missions, where it was shown that gravity is not primarily responsible for the induction and maintenance of circumnutations [25,26].

The interest for sunflower phototropism has been recently renewed, mostly in connection with the sun-tracking ability of sunflower. Only the uppermost shoot internode bearing the inflorescence has a sun-tracking ability. During the day, the shoot apex continuously

reorients its position, allowing the heads to track the relative position of the sun from east to west [27]. At night, inflorescences reverse their daytime movement and turn to point towards the east, providing a synchronized starting position at dawn. Sun-tracking stops at some point after anthesis, and the inflorescences then remain arrested, pointing to the east. Inflorescence sun-tracking has been studied recently [28-30], providing a complex network of regulatory factors with a prominent role of circadian regulation.

Our study aimed to investigate how photoperiods establish and affect the diurnal rhythmicity of PT bending responses in sunflower seedlings. Our focus was on darkness, which is not an absolute requirement for the establishment of a PT bending response, but which affects the order of daily events within photoperiods. We will show that the PT bending capacity of plants is restored and synchronized in the darkness (dark period, night, nighttime) compensating for the large differences that exist in the duration of daytime in some photoperiods. The diurnal rhythms we studied were fixed by the growth chamber to last 24 h and were considered as circadian rhythms. Diurnal (circadian) rhythmicity is a consequence of circadian regulation, which controls the expression of numerous genes comprising the plant genome [31]. It helps plants to synchronize major metabolic processes, such as photosynthesis and respiration, with the optimal times of the day or night. True circadian rhythms persist for days in free-running (constant) conditions and this is the main criterion used for their validation [32]. In potato shoot culture, circadian regulation of PT bending was questionable due to its rapid damping [33], which we checked here in sunflower, observing that even faster damping occurs in seedlings entering constant light conditions.

MATERIALS AND METHODS

Seed germination and plantlet cultivation

The study was performed with seeds of *Helianthus annuus* L. cv. Kondi (Syngenta), widely used in field production in Europe. Germination was initiated by washing and submersing seeds in tap water (imbibition) for 12 h. Germination ensued after placing the seeds in enclosed trays beneath several layers of moist tissue paper. Seeds were inspected and sorted 24 h

later. Those with a 2-5 mm-long radicle were sown individually in 50-mL polypropylene centrifuge tubes filled to the rim with a fine-sieved, well-watered peat-based substrate. Imbibition and sowing procedures were performed under dim white light $2.0\text{-}2.5\ \mu\text{mol m}^{-2}\text{s}^{-1}$ irradiance at $24\pm 1^\circ\text{C}$. The start of imbibition and start of daytime (dawn) in the growth chamber were synchronized in all investigated photoperiods, which allowed for the night points to be shifted and investigated during standard working hours of the laboratory. After sowing, the tubes with germinating seeds were placed in growth chambers (Frigoline-Star, Serbia) adjusted to maintain a $24\pm 0.5^\circ\text{C}$ temperature for all specified photoperiods. Light at $70\ \mu\text{mol m}^{-2}\text{s}^{-1}$ irradiance was provided by Philips TL-D lamps and photoperiod duration was adjusted by timers. Irradiance of all light sources was measured by a Li250A light meter equipped with a quantum sensor.

Photoperiods

A long day, 16/8 h light:darkness, was used as the initial photoperiod for optimization of experimental protocols and to track how developmental changes affected phototropic (PT) bending of plantlets. The five diurnal photoperiods that were studied in detail included those with 8/16, 10/14, 12/12, 14/10 and 16/8 h of light:darkness. In all of them, the beginning of daytime (dawn) was adjusted to start at the same time, 120 h after seed imbibition.

Free-running conditions included plantlets grown in constant light (CL) and constant darkness (CD). Both free-running conditions could be provided simultaneously in the growth chamber by using light insulated boxes for CD and keeping the lights constantly turned on for CL conditions. Seeds were exposed to free-running conditions from the start of imbibition, except for the sowing, which was done under dim light ($2.0\text{-}2.5\ \mu\text{mol m}^{-2}\text{s}^{-1}$).

For experiments with prolonged light duration, light was not turned off at the end of the last day before the start of PT bending stimulation. Conversely, in prolonged night duration, light was not turned on at the end of the last period of darkness. Prolonged night or daytime duration experiments can be considered as delayed light transitions that mimic the start of free-running conditions.

Phototropic stimulation

PT bending was followed by placing plantlets in the beam of unilateral light produced by blue LED lamps. Experiments were performed in dark chambers (80x60x40 cm) lined with black cardboard. The illuminating assemblage consisted of either a single or two adjacent LED Phillips GU10 (1 W) narrow beam accent lamps. They created a circular area of light of $16\text{-}24\ \mu\text{mol m}^{-2}\text{s}^{-1}$ irradiance when placed 30-35 cm from the plantlets. Lamps had a peak emission at 465 nm as measured by an Ocean 2000 NIR-UV spectrophotometer. Single lamps could illuminate the apical portions of 3-4 plantlets and a pair of adjacent lamps illuminated 5-6 plantlets. Blue-light PT stimulation lasted continuously for 4 h. Plantlet PT bending was recorded using the time-lapse feature of Nikon P510 and Nikon P520 cameras, which were adjusted to take images at 10-min intervals. The cameras were positioned transversely to the PT bending directions of the plantlets. The blue light produced by the LED lamps was sufficient to provide images of good quality.

Phototropic bending treatments

Groups comprising 3-6 plants sampled from the growth chamber at prearranged times were promptly mounted in a dark chamber and exposed to blue light to induce PT bending, which was then recorded by a time-lapse technique for the next 4 h at 10-min intervals. Every group, referred to here as a PT bending treatment, had a collection of 24 images that were stored with the exact date and timing available for every image of the collection. PT bending treatments for a certain photoperiod and the time of day at which plantlets were sampled were replicated 5-8 times, allowing for subsequent statistical analysis of groups of 24-30 plantlets sharing the same sampling time within a photoperiod. Time-points are the basic units for which average PT bending magnitudes were calculated. For every time-point, a graphic presentation of PT bending magnitudes was plotted in 10-min increments. A complete list with all time-points of PT bending and for all the diurnal photoperiods that were studied here is presented in Supplementary Table S1. The arrangement of plantlets for PT bending stimulation and for measuring the PT bending angle α is presented in Fig. 1A.

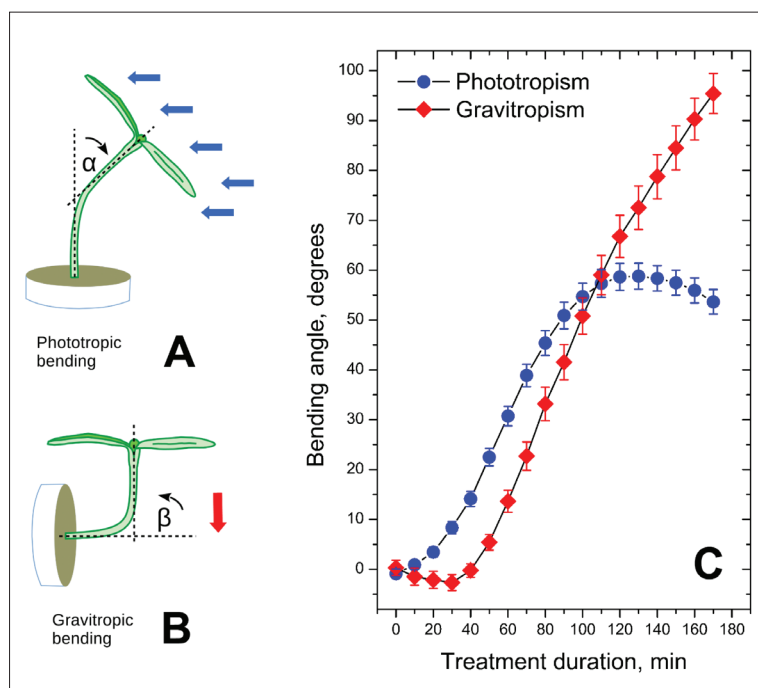


Fig. 1. **A** – Schematic presentation of a plantlet at the end of the PT bending with bending angle marked as α . Blue arrows indicate incoming unilateral light. **B** – For GT bending, the plantlets were placed with shoots positioned horizontally; red arrow indicates the direction of the earth's gravitational force. By the end of the GT bending the upper shoot portions always attained a vertical position. The GT bending angle marked as β often reached angles larger than 90° , indicating transient overshoot of the vertical position. **C** – PT and GT bending of sunflower hypocotyls compared side by side. Plantlets grown in 16/8 h light:darkness mounted for bending 6 h after dawn on the 6th day after imbibition. PT bending started earlier but after 60-70 min the bending rate began to decline enabling GT to overcome PT bending. The values are averages with standard errors of the mean; $n = 25$ plantlets. PT – phototropic, GT – gravitropic

Gravitropic bending treatments

Gravitropic (GT) bending was studied briefly as it did not manifest diurnal rhythmicity for GT bending like PT bending. GT stimulation was induced by placing plantlets in a horizontal position. GT bending was recorded by a transversely positioned camera at 10-min time increments, like the PT bending. GT bending was performed in the dark but digital imaging required the plantlets to be illuminated for 5-6 s every 10 min. Illumination was done with amber-colored Philips LED 1W GU10 narrow beam accent lamps producing a dim light of $2.0\text{--}2.5 \mu\text{mol m}^{-2} \text{s}^{-1}$. The amber light did not induce bending responses in the plantlets. The arrangement of plantlets for GT bending stimulation and for measuring the bending angle β is presented in Fig. 1B.

Parameters of phototropic bending

In addition to the PT bending magnitudes that were calculated as averages of individual plantlets comprising a time-point, we used some indirect PT bending parameters calculated from the data used for plotting the PT bending curves. They included Lag phase duration, Slope (rate) of PT bending and the time available for bending (TAB). Lag phase duration was the time required by shoots to perform a visible 2.5° initial increase of their PT bending angle. The slopes were measured at the inflection point of PT bending curves. The TAB was calculated by subtracting the lag phase duration from the time the plantlets required to reach the highest value for PT bending magnitude in a time-point.

Data management and statistics

Values of PT bending magnitudes measured by GIMP 2.8 (www.gimp.org) for individual plantlets within the same time-point were arranged and stored in spreadsheets of OriginPro 8 (www.OriginLab.com). Their average values, calculated in 10-min increments, were plotted as graphs by OriginPro 8, and they were further analyzed or used to calculate indirect PT bending parameters.

After the PT bending treatments, the plantlets were discarded as each plantlet was used only once in the study. One-way ANOVA followed by Tukey's HSD test were used for comparisons of the means measured for highest PT bending magnitudes of diurnal photoperiods [34].

RESULTS

The phototropic (PT) and gravitropic (GT) bending responses of sunflower seedling hypocotyls are compared in Fig. 1C. The PT bending response had a conspicuous maximum, after which the bending angle declined and the shoot straightened. The GT

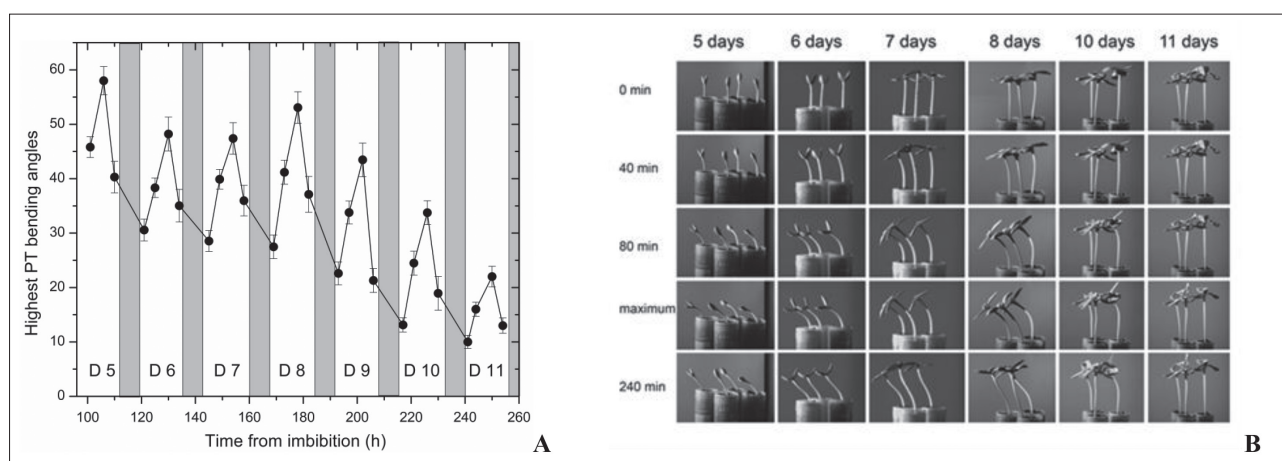


Fig. 2. A – Diurnal changes of PT bending magnitudes in hypocotyls grown in 16/8 h light:dark photoperiod during 7 consecutive days, corresponding to the period lasting 100-250 h after the start of imbibition. Daily pattern repeats day by day gradually declined and expired 11 days after imbibition. The values are averages of PT bending magnitudes with standard errors of the mean; $n = 27$ plantlets. **B** – PT bending of plantlets at midday for each successive day.

response always ended with an apical shoot portion reaching a complete 90° bending angle, which was then maintained with some transient overshoot. The lag phase duration of the GT bending response took more time than in PT bending and therefore it started later. However, because the GT bending response is stronger it overtakes the PT bending. This is why standard, unilateral PT bending in sunflower seedlings never reaches the full 90° bending angle [15]. The GT bending of sunflower hypocotyls has a limited diurnal rhythmicity, the same as in the case of potato shoots [33], and it was not studied further.

Developmental changes

The PT bending ability of sunflower seedlings was established as soon as the hypocotyl appeared above the soil surface on the 5th day, lasted for the next seven days and then gradually started to decline (Fig. 2). On each day, the highest PT bending magnitude was achieved at midday, with minimal values observed at nighttime close to dusk. PT bending was exceptionally high when the cotyledons of germinating seedlings started to open, usually on the 5th day of germination about 100 h after the start of imbibition. Seedlings also manifested a transient increase in PT bending ability 8 days after the start of imbibition. Otherwise, from the time of cotyledon opening and onward, the PT bending ability of the hypocotyl steadily declined and ended 11 days after the start of imbibition (Fig. 2A, B);

by this time, the PT bending ability of the hypocotyl was transferred to the uppermost, rapidly developing shoot of the first internode.

Diurnal photoperiods

The PT bending magnitudes of the hypocotyl changed significantly throughout the day in all diurnal photoperiods. These differences were visible both in the magnitudes of PT bending and in the shape of the graphs presenting the PT bending process. For the 14/10 and 8/16 h light:darkness photoperiods, the changes in daily PT bending are presented in detail for all time-points (Fig. 3A-D). Differences in the maximal PT bending angles achieved by plants in the 14/10 photoperiod ranged from $67.7 \pm 2.1^\circ$ in the time-point starting 6 h after dawn, to $25.9 \pm 1.4^\circ$ in the time-points starting 4 h after dusk. For the short-day 8/16 h photoperiod, the differences were also significant, ranging from $66.3 \pm 2.7^\circ$ during daytime to $11.9 \pm 1.7^\circ$ during nighttime.

Starting at dawn, PT bending magnitudes in all diurnal photoperiods rapidly increased and reached the highest daily values about midday. PT bending then gradually declined until the end of daytime, at dusk. PT bending declined further with the start of nighttime until a nighttime minimum was reached at time-points starting 4 h after dusk. Only in the long-day 16/8 h photoperiod was the nighttime minimum positioned

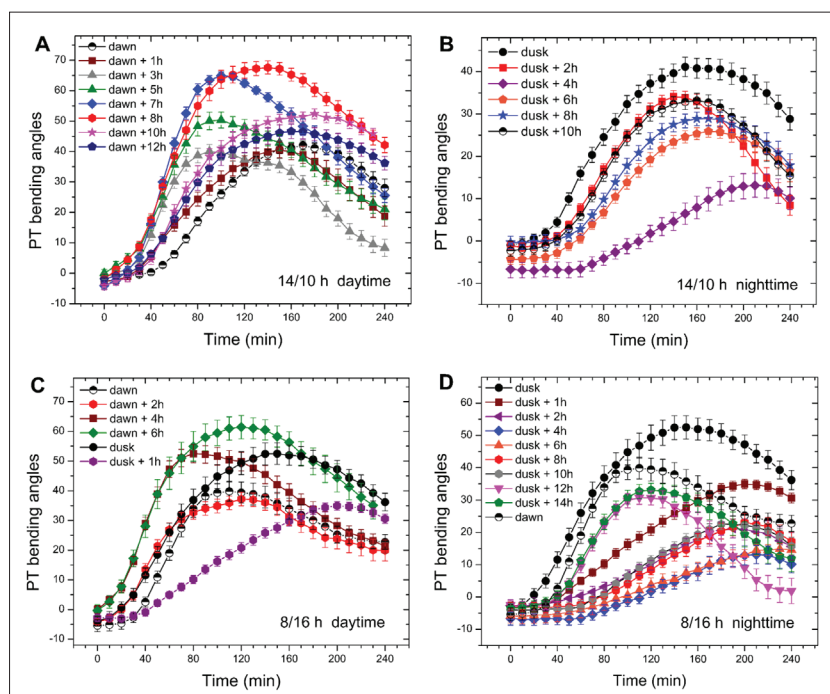


Fig. 3. Changes of the PT bending magnitudes in time-points of diurnal photoperiods in the 6th day, 120-144 h after imbibition). **A** – Daytime (dawn to dusk) time-points 14/10 h LD photoperiod. **B** – nighttime (dusk to dawn) time-points 14/10 h LD photoperiod. **C** – Daytime (dawn to dusk) time-points 8/16 h LD photoperiod. **D** – nighttime (dusk to dawn) time-points 8/16 h LD photoperiod. The values are averages of PT bending magnitudes with standard errors of the mean; n – 24-30 plants per time-point.

2 h after dusk. From the nighttime minimum, the PT bending magnitudes increased again until dawn.

The differences in the timing (position) of the PT bending maxima along the X-axis in time-points starting at different times of day were conspicuous and indicative as they affected the shape of the PT bending curves. At time-points around midday, the maxima of the PT bending magnitudes were observed 80-120 min from the start of PT stimulation. At time-points close to dusk, the same as in those starting in darkness, the maxima of the PT bending magnitudes were shifted and were observed 200-220 min after the start of PT stimulation. Transposed into TAB parameter values, it was clear that plantlets at time-points starting during nighttime had much more time available for PT bending than in samples in which the time-points started in daytime. However, the rate of PT bending at time-points that started around midday was much faster than in those starting in nighttime (Fig. 3A and B, Fig. 3C and D). Therefore, the longer time available for bending at time-points starting during nighttime compensated somewhat for their lower bending rates.

In the 8/16 h light:darkness photoperiod with a long nighttime, the bending magnitudes of the plantlets started to increase during nighttime, considerably before the light transition at dawn was reached (Fig. 3B). They were apparently compensating for the short duration of day in this photoperiod, enabling the PT bending magnitudes to reach high values during the short daytime. In the 16/8 h light:darkness photoperiod with short nighttime, the PT bending magnitudes at the end of nighttime increased only if the duration of night was prolonged. This was observed when plants, instead of exposure to light transition at dawn remained in darkness longer. The highest nighttime PT bending capacity was observed when nighttime duration was extended to last for 4 h more (Fig. 5B).

Comparison of PT bending between diurnal photoperiods

Comparison of PT bending in plants grown in different diurnal photoperiods was performed by plotting only the single highest PT bending magnitude for every time-point within a photoperiod (Fig. 4A). The resulting sinusoid curves had a maximum around midday and a minimum at nighttime, 4 h after dusk. Similarly, the shortest lag phase durations of all time-points in the five diurnal photoperiods are presented in Fig. 4B. The sinusoid curve of lag phase duration is inverted in relation to the sinusoid curve of the PT bending magnitudes, with the highest values noted at night and the lowest values during daytime.

The statistical significance of the highest and lowest daytime PT bending magnitudes registered in Fig 4A for the 5 diurnal photoperiods is presented in Table 1. The connection between PT bending magnitudes and daytime duration is not consistent. It is more apparent for the points comprising the minima of the sinusoid curves. The connection is less apparent

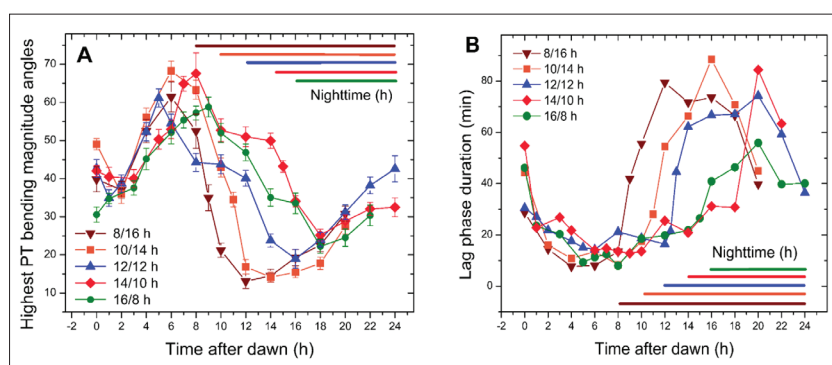


Fig. 4. Comparison of the highest PT bending magnitudes in time points of 5 diurnal photoperiods (A), and the durations of their lag phase (B) measured on the 6th day after the start of imbibition. Horizontal lines indicate the start and duration of night. The values are averages and standard errors of the mean for PT bending magnitudes. Lag phase duration data of (B) was calculated from the average PT bending magnitudes of time points (A) and therefore have no standard errors. All presented time points of 5 photoperiods; n – not less than 24 plantlets per time point.

Table 1. Statistical test of highest and lowest daily phototropic (PT) bending magnitudes registered in time-points of 5 diurnal photoperiods presented in Fig. 4A. The test shows significant difference between the means and a stronger connection between daytime duration and PT bending magnitudes for the lowest PT bending magnitude values than for the highest PT bending magnitudes.

photoperiod*	lowest PT bending magnitudes		highest PT bending magnitudes	
8 / 16	11.89±1.71 a		66.35±2.71 ab	
10 / 14	18.45±2.52 ab		68.23±1.23 b	
12 / 12	18.94±2.52 ab		61.24±2.85 ab	
14 / 10	25.92±1.42 b		67.60±2.14 ab	
16 / 8	25.42±2.23 b		58.80±2.60 a	
ANOVA source of variation	df	Mean square	F	P
lowest magn.	4	858.11	8.0951	0.000008
highest magn.	4	457.3	3.281	0.013707

One-way-ANOVA followed by Tukey's multiple comparison test at $P < 0.05$. *photoperiod = daytime hours/nighttime hours.

for the highest daytime PT bending magnitudes observed around midday. In other words, it seems that the highest daily magnitudes of PT bending in different photoperiods were buffered and equalized. This enabled the plants to maintain and express high PT bending responses during daytime in all diurnal photoperiods, irrespective of the large differences existing in daytime duration.

An important finding that can be seen from the curves presented in Fig. 4A is that the lowest

(minimal) daily values of PT bending magnitudes in all photoperiods were recorded in time-points that started 4 h after dusk, with a single exception for the long-day 16/8 h photoperiod. It appears as if there was a kind of recovery process working on PT bending capacity (bending magnitudes) that required the plants to spend a certain number of hours in darkness. However, the study of plants grown under CL conditions indicated that darkness *per se* was not an absolute requirement for the development of a strong PT bending response.

The lag phase durations presented in Fig. 4B also point to the recovery points located in nighttime, but their positions seem to relate to the duration of the previous daytime and not to the duration of darkness. Following the recovery point, the ability of plantlets to perform PT bending constantly improved until daytime.

Phototropic bending in constant light

Plantlets grown in CL manifested a PT bending curve that was the same in shape and PT bending magnitudes for all PT bending treatments performed during this everlasting day. Since there were no differences between the time-points that started at different times of the day, PT bending under CL was considered as uniform, contrasting with the rhythmic daily PT bending observed in diurnal photoperiods.

A characteristic feature of the PT bending curves in CL plants was the combination of a short duration of the lag phase, high PT bending rates and short TAB durations. This unique combination provided a truncated-like appearance to the graphs of PT bending under CL, resembling the PT bending responses in the early daytime time-points of diurnal photoperiods.

In plants grown in CL conditions, the uniform CL pattern of PT bending was visible as soon as the hypocotyls emerged above the soil surface, indicating its early and rapid establishment. Indeed, in plants

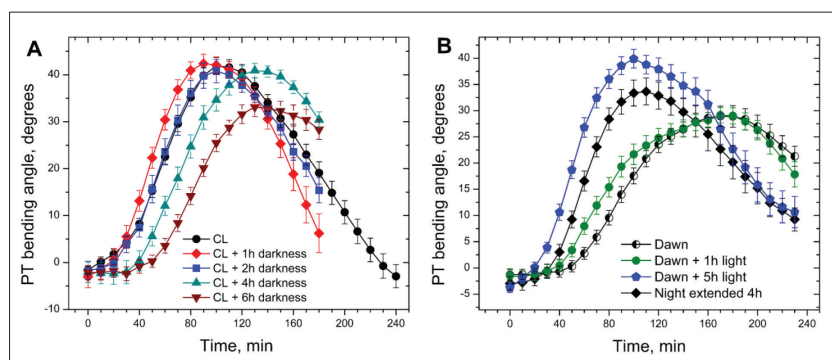


Fig. 5. Effects of darkness on the magnitudes of PT bending. **A** – Plantlets grown under constant light (CL) from the time of imbibition and darkness applied by turning the lights off on the 6th day. PT bending was measured after 0, 1, 2, 4 and 6 h of darkness. Decrease in PT bending magnitudes is first observed and coupled with extended lag phase duration after 4 h of darkness. **B** – In the 16/8 h light:dark photoperiod, night is 8 h; extending the duration of the last night significantly promotes PT bending. The effect is most prominent if nighttime (darkness) is extended to last 4 h more to resemble the values registered in daytime. The values are averages with standard errors of the mean; n – not less than 24 plantlets per time point.

grown under diurnal photoperiods, the absence of a single night at the end of a day (prolonged day duration) was sufficient to cancel rhythmicity and induce the appearance of a uniform CL type of PT bending. Thus, plantlets grown in diurnal rhythms could not maintain rhythmicity of PT bending under CL, indicating a possible malfunction of circadian regulation.

Plantlets developing in CD were etiolated. They manifested the total absence of PT bending ability, thus limiting the use of CD in studies of phototropism in sunflower seedlings. A fast but incorrect conclusion here would be that darkness is a condition detrimental for PT bending. Sunflower plantlets do not need darkness to establish their PT bending responses, but it apparently helps them to synchronize their responses in diurnal photoperiods.

We have seen that in the short-night 16/8 h light:darkness photoperiod, prolonging the duration of the previous night transiently improved PT bending. But if night duration is prolonged to last more than 4 h, plants will interpret this change as the start of constant darkness (as the CD condition), inducing a progressive deterioration of PT bending capacity.

When the CL free-running condition ended by the start of a dark period (by turning the lights off), the CL type of PT bending response persisted for just a couple

of more hours. After the first 4 h of darkness, a prolonged duration of the lag phase was observed while a decrease in PT bending magnitude was visible after 6 h of darkness (Fig. 5A). Therefore, the decline of PT bending magnitudes in CL grown plants triggered by darkness resembled the decline induced by prolonging the duration of the last night in diurnal photoperiods.

DISCUSSION

The PT bending ability of sunflower hypocotyls is a short-lived feature affected by a number of different factors. Using controlled conditions in a growth chamber we

could neutralize and cancel the effects of light intensity, temperature and water availability, and study how exposure to light and its daily duration affected PT bending.

The presence of light seems to be an absolute necessity for the initial establishment of the PT bending response of hypocotyls, since in etiolated seedlings it was absent and appeared only upon exposure to light (de-etiolation). But periods of darkness that occur within diurnal photoperiods have a completely different role. They enable (or reflect) synchronization with other metabolic processes resulting in the establishment of diurnal rhythmicity of the PT bending capacity. Thus, periods of darkness are far from being detrimental, they provide suitable conditions for plantlets to progress through metabolic pathways that differ from those occurring in daylight, as was shown in potato [35]. Therefore, periods of darkness in diurnal photoperiods appear to be quite useful to plants. This necessity for darkness in sunflower seedlings is best observed in the case of the lowest PT bending, which in almost all photoperiods occurred at time-points starting after 4 h of darkness (Fig. 4A). Conversely, in photoperiods with a short night duration (16/8 h light:darkness), prolonging the duration of the last night from 8 to 12 h was beneficial as it improved the PT bending magnitudes.

PT bending experiments performed in continuous light (CL) revealed the other side of the light-darkness

relation. Darkness, considered as the absence of light, is not an absolute requirement for PT bending. But the PT bending magnitude in time-points starting at midday of diurnal photoperiods were higher and the bending lasted longer than in experiments done under CL. The increase in PT bending in diurnal photoperiods can hardly be described as advantageous. It just seems that the capacity for PT bending in diurnal photoperiods has a better daily distribution.

The daily maximum of PT bending magnitudes in diurnal photoperiods falls around midday, but this synchronization actually goes back to the previous nighttime in which the low PT bending occurring at night acts as a common synchronization point. When a new day starts, plants already anticipate how long it will last. The short time that elapses between dawn and the daytime PT bending maximum at midday in the short-day 8/16 h light:darkness photoperiod is actually the consequence of the increase in PT bending capacity, which already started at nighttime, long before dawn came. Thus, it seems that plants in their calculation of the length of day for the requirements of PT bending use a reference system that is different and more complex than a simple determination of the duration of daytime or nighttime. The question of daytime and nighttime length determination in plants is one of the most elaborated problems of plant biology, usually studied in relation to flowering, with little data obtained from studies of phototropism.

In potato, only the long-day 16/8 h light:darkness photoperiod is suitable for studies of phototropism as in the short-day photoperiod, potato cultures are prone to form light insensitive stolons and tubers instead of true leafy shoots [36]. In *Arabidopsis*, the problem is different as light inhibits shoot elongation and development [37], allowing for studies only in short days, not those in other diurnal photoperiods with longer days. Therefore, sunflower seedlings that grow very well in a variety of different diurnal photoperiods are the material of choice for studies of phototropism in different diurnal photoperiods.

Sunflower seedlings possess a unique feature because under prolonged daytime duration they readily slip from the rhythmic PT bending of diurnal photoperiods into the uniform PT bending of CL. The absence of PT bending in darkness prevents us from

using a similar experimental setup with extended duration of nighttime. The improved PT bending observed in the extended night duration of the long-day 16/8h light:darkness photoperiod is just a transient feature lasting no more than 4 h.

The rapid transition of PT bending from a rhythmic event in diurnal photoperiods to uniform, arrhythmic PT bending after prolonged duration of daytime indicates that circadian regulation of PT bending is arrested under free-running conditions. This malfunction of circadian regulation can perhaps be considered as a programmable adjustment or some kind of safety valve. The circadian regulation of PT bending in sunflower seedlings needs further study.

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Supplementary Material

The Supplementary Material is available at: http://www.serbiosoc.org.rs/NewUploads/Uploads/Vinterhalter%20and%20Vinterhalter_6234_Supplementary%20Material.pdf