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Circadian rhythms of the L-ascorbic acid level in *Euglena* and spinach

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**Key words**: antioxidant, L-ascorbic acid, circadian, *Euglena gracilis*, midday, photooxidation, spinach

**Abbreviations**: Asc, L-ascorbic acid; CT, circadian time; DH-Asc, dehydro L-ascorbic acid; DD, continuous darkness; GT, average generation time; LD:x,y, (x + y) h light/dark cycles with x-h light; LL, continuous light; PET, photosynthetic electron transport; ROS, reactive oxygen species.
Abstract
Plant defenses against photo-oxidative stress have been studied almost exclusively with respect to stress responses, and little is known about how non-enzymic antioxidants change under constant conditions without a time cue or an environmental stress. Here, we show that, in both the flagellated alga *Euglena gracilis* Z and the angiosperm *Spinacia oleracea* L., the potent antioxidant L-ascorbic acid (Asc) displays a circadian rhythm with a maximum at subjective midday, a physiological state reflecting that attained at noon under daily light/dark cycles. Thus, photosynthetic organisms can maximize antioxidant levels in anticipation of midday, when photo-oxidative stress is most severe. These results may partly explain the in-phase circadian UV-C resistance rhythm recently identified in the alga. However, the Asc, but not the resistance, rhythm wanes in continuous darkness. This suggests the presence of persistent circadian rhythms in the levels of other antioxidants in continuous darkness, which may account for the UV-C resistance rhythm.

1. Introduction
The circadian rhythm is a biological mechanism for anticipatory adaptation to the daily and seasonal variations in the earth’s environment. It can persist, or free-run, with a period of about 24-h in environments lacking external time cues. It can also respond to and thereby run in phase with, or entrain to, 24-h light/dark cycles (LD), such that a subjective day, which is an endogenous state reflecting what is attained in the light interval during 24-h LD cycles, occurs exactly during the light. Circadian rhythms are driven internally, but the phase can be reset by external time cues.

In the algal flagellate *Euglena gracilis*, numerous aspects of circadian rhythm have been studied [1, 2], including the biochemical mechanisms for autonomous oscillation [3], the physiological mechanisms for gated cell population growth [4], and the photoperiodic induction of cell reproduction [5]. It is generally believed that most if not all rhythms have adaptive significance, and in some cases reasonable possibilities have been suggested [6]. In *Euglena*, circadian rhythms in phototaxis and photosynthetic capacity that peak at subjective midday may play a role in maximizing the use of solar-energy, which is highest at noon [2]. However, this peak in solar energy also threatens to photo-oxidize photosynthetic organisms. According to our ‘resistance to light’ hypothesis, harmful noon-time solar radiation, both visible and UV, would have killed photosynthetic organisms that had not developed a mechanism for midday maximization of defenses against such radiation-induced stress [7]. Accordingly, we found UV-resistance rhythms peaking at subjective midday [7]. The circadian G2-to-M
phase arrest [4] may also provide similar protection, but in a different manner, according to the ‘escape from light’ theory [8-11]. The rhythm relegates cells to M phase, the cell cycle phase most sensitive to UV and ionizing radiation, during the night intervals. Moreover, photoperiodic cell reproduction may play a role in seasonal adaptation in this alga [5].

We have previously shown that circadian UV-resistance rhythms may involve non-enzymic antioxidants. Supporting evidence comes from the observation that UV resistance, as judged by resistance to immediate cell death after a short exposure to UV, is insensitive to temperature and increases when non-enzymic antioxidants are applied exogenously. Resistance to UV-C or UV-B was increased by the application of L-ascorbic acid (Asc) or dimethylsulfoxide (DMSO), respectively [7]. However, little is known about whether a particular antioxidant displays a circadian rhythm, although diurnal rhythms under 24-h light/dark (LD) cycles have been demonstrated for β-carotene levels in the dinoflagellate Gonyaulax polyedra [12] and for Asc levels in Euglena gracilis [13].

The driving force behind these diurnal antioxidants’ rhythms may be the cell division cycle or a circadian rhythm, or the oscillation may be a direct response to external cycles. Knowledge of the driving force will elucidate the processes’ biological role and mechanism of action. Here, we examine the circadian component of the ‘resistance to light’ hypothesis. The ‘resistance to light’ hypothesis predicts circadian rhythms for various antioxidants. We postulated that Asc levels display a circadian rhythm, based on our previous results suggesting a role for Asc in the circadian UV-C resistance rhythm of E. gracilis. As the ‘resistance to light’ hypothesis involves all photosynthetic organisms and is not restricted to a particular species such as Euglena, we extended this study to spinach, for which a circadian rhythm has not yet been documented.

2. Materials and methods
2.1. Organisms

Euglena gracilis Klebs (strain Z) was cultured at 25°C in modified Cramer-Meyer medium [13] without organic substrate. The cultures were irradiated unilaterally from an array of cool white fluorescent lamps at an incident luminance of 6 klx (84 μmol m⁻² s⁻¹). The alga was first grown under continuous light (LL) and then transferred to either a 24-h LD cycle with a 12-h photoperiod (LD:12,12), a LD:1,1 cycle, or continuous darkness (DD). In some cases, the culture bottles were wrapped in yellow plastic membrane (Rosco Supergel #10) to eliminate blue light, reducing the light intensity at the culture
surface to 80% that of the unwrapped bottles. Approximately 7 ml of \textit{Euglena} culture were withdrawn automatically every 2 h and fixed with 0.5 ml of 20% neutral formalin containing 5% KCl. The cell number was counted with a Coulter Electronic Particle Counter.

The seeds of spinach, \textit{Spinacia oleracea} L., were purchased from Sakata Seed Co. (Japan) and cultivated in a growth room at constant temperature, either 20°C or 25°C, and 50% relative humidity. The spinach was germinated under LD:12,12 at a luminance of either 30 or 20 klx at the soil surface. Thirty-five days after germination, the spinach was transferred to LL and then harvested to determine the Asc content.

2.2. Circadian time (CT) definition in non-dividing cultures

The time of the transfer from LL to DD was defined as CT12 [1, 2]. According to our previous study, the period of the circadian rhythm for the photo-induction of the commitment to cell division in \textit{E. gracilis} is approximately 26 h [5]. Thus, CT12 was considered to have occurred at the following hours in DD: zero, 26, 52, 78, and 104 h. When the cells were transferred from LL to LD:1,1, the onset of cell population growth, or CT12, would have occurred, on average, at the following hours: 22, 48, 74, and 100 h [4]. The present study assumed that CT12 in non-dividing cultures also occurred at these times in LD:1,1.

In spinach, the onset of LL (the end of the dark period of the last LD: 12,12) was CT00, which is thought to recur at 24-h intervals thereafter, given that the circadian period, at our experimental resolution, was not significantly different from 24 h.

2.3. Extraction and determination of Asc and dehydro (DH)-Asc

2.3.1. \textit{Euglena}

\textit{Euglena} cells (10-20 \times 10^6) were collected every 4 h by centrifugation at 460 \times g for 10 min at 4°C. The cell pellet was suspended in 10 mM potassium phosphate buffer (pH 6.0) and centrifuged again. The cells were washed again with centrifugation at 13,000 \times g for 10 min, and the resultant cell pellet was stored in liquid nitrogen for later use.

The frozen cells were rapidly thawed, suspended in 0.6 ml of 0.5% metaphosphoric acid, and subjected to sonication for 10 s at a power of 36 W with a 10% duty cycle. The sonication was performed in an ice-bath and was repeated 12 times, at intervals of 20 s for cooling. The homogenate was centrifuged at 24,000 \times g for 10 min, and the supernatant was used to determine the Asc and DH-Asc contents using the 2,4-diphenylhydrazine method, essentially similar to that described in [13].
One batch of culture was used for each time series, and two frozen cell samples were prepared for each time point (4-h intervals) over one to three circadian cycles. The Asc and DH-Asc assays were performed once for each extract. The results are presented as 3-point moving averages for each time series, as well as raw data.

2.3.2. Spinach

Spinacia leaves, 35-37 days old, were used. At this age, the leaves were no longer increasing in length, and the cell cycle should have terminated. Two leaves were harvested every 4 h for 48 or 72 h. Each blade (0.2 to 0.5 g) was separately frozen and macerated with a mortar and pestle in liquid nitrogen. To each macerated leaf, we added 10 vol (2 to 5 ml) of 3% metaphosphoric acid, followed by freezing with dry-ice acetone and storage at –40°C for later use.

The frozen material was rapidly thawed and centrifuged twice at 10,000 × g for 15 min to precipitate any residue. The supernatant was passed through a membrane filter of 0.45-µm pore size. Ten µl of the clear filtrate were subjected to HPLC on a Shinwa ULTRON-PS-80H column using dilute perchloric acid (30 µM, pH 4.0) as the solvent, at a flow rate of 1.0 ml/min; the absorbance of the eluate was detected at 254 nm. The Asc content was estimated based on the chromatogram, with the aid of a Shimadzu Chromatopac C-R4A. Two independent measurements were made for each extract.

3. Results

3.1. Euglena

A very slowly dividing culture of Euglena, with a daily increase of cell number of 10% [i.e., an average generation time (GT) of 175 h], displayed a circadian rhythm of intracellular levels of Asc and total Asc (Asc+DH-Asc) that was entrained to LD:12,12 (Fig. 1A). The amplitude (0.6 to 3.2 nmols per 10^6 cells) was comparable to that (1.8 to 4.2 nmols per 10^6 cells) reported by Shigeoka et al. [13] in a synchronous culture (factorial increase, 2.0; GT, 24 h), indicating that the rhythm was independent of cell cycle progression, although the mean level was somewhat lower in our culture. The Asc content represented more than 80% of the total Asc content, and there were no apparent rhythms in the redox state of Asc, based on the ratio of Asc to Asc+DH-Asc.

Figure 1B was obtained by calculating the difference between each two consecutive values for the total Asc plotted in Fig. 1A. A circadian rhythm of the net increase in the total Asc level was identified (Fig. 1A), with a maximum at around subjective dawn. Little Asc or DH-Asc was present in the extracellular medium [15], and thus the net increase represented net synthesis of Asc. Therefore, the circadian rhythm of the net
synthesis of Asc, with its maximum at around dawn, determined the rhythm of the total Asc level.

When the algal cultures under LL were transferred to white LD:1,1 (Figs. 2 and 3), yellow LD:1,1 (Fig. 4), or DD (Fig. 5), they manifested circadian rhythms in the Asc level and in the net synthesis of Asc; LD:1,1 does not serve as a time-cue and has often been used as a free-running condition particularly to study circadian timing of cell division in the alga [2, 4]. Obviously, the driving force of these rhythms was not an external 24-h LD cycle. Whether cells divided (Fig. 2) or not (Figs. 3 to 5), the circadian rhythms persisted. Specifically, we found comparable amplitudes and mean levels in the cultures of dividing (Fig. 2) and non-dividing (Fig. 3) cells in LD:1,1. Thus, as for the rhythm in LD:12,12 (see above), cell cycle progression played no role.

Within the time range used in the experiments testing UV-C and UV-B survival rhythms [7], maximum Asc levels were attained between hours 63 and 69 in LD:1,1 (Figs. 2A, 3A, and 4A), which was close to the maximum (hours 66 to 70) of the UV resistance rhythm. The minimum Asc level occurred between hours 53 and 55 in LD:1,1 (Figs. 2A, 3A, and 4A), corresponding to CT15 to CT17. This was very close to, but not exactly matching, the minimum (hours 56 to 58) of the UV resistance rhythm [7].

The amplitude and mean level of Asc were highest in LD:1,1 (Figs. 2 and 3), and they were decreased by half in yellow LD:1,1 (Fig. 4). Yellow light also reduced the rate of cell population growth (data not shown) to about half that in white LD:1,1. As the growth rate depends on the activities of photosynthetic electron transport (PET) [5, 16], the effect of yellow LD:1,1 was probably the result of reduced PET. The amplitude and mean level of Asc were extensively reduced in DD (Fig. 5), perhaps owing to the depletion of Asc, which in turn may have resulted from the depletion of the starting material for de novo synthesis, which would have been provided by photosynthesis.

Shigeoka et al. [15] reported that blue light, but not other component spectra of visible light, increases the Asc+DH-Asc level in dark-adapted E. gracilis grown heterotrophically. If this were true in our photoautotrophic culture, the temporal change of the Asc level in yellow LD:1,1 (Fig. 4), where blue light was totally excluded, would have been similar to that in DD (Fig. 5). However, this was not the case. The difference in the response to light between photoautotrophic and heterotrophic Euglena is also apparent in the effect of light on cell cycle progression; light arrests the cell cycle in heterotrophic Euglena, whereas darkness arrests the cell cycle in photoautotrophic Euglena [16, 17].

3.2. Spinach
A circadian rhythm of Asc content was also found in the leaf blades of spinach. The entrained rhythm, with a maximum around subjective midday, was expressed in spinach raised under LD:12,12 at either 25°C/30 klx or 20°C/20 klx (data not shown). When the plants were then transferred to LL, at either 25°C/30 klx or 20°C/20 klx, they displayed a free-running rhythm, with a maximum around subjective midday (Fig. 6), as in *E. gracilis* (Figs. 1 to 5). As for other circadian rhythms [1, 2] the period length was close to 24 h and relatively constant at different temperatures, light intensities and growth rates. It was slightly shorter at 25°C than at 20°C.

4. Discussion

The intracellular level of Asc displayed a circadian rhythm, with a maximum occurring at around subjective midday, in both *E. gracilis* and *S. oleracea*. This is the first report on circadian rhythms of non-enzymic antioxidant levels in photosynthetic organisms. In both cases, the rhythm persisted not only independently of but also entrained to the external time cue (24-h LD cycles). Moreover, the temperature independence of the period was shown in *S. oleracea*. In the case of *Euglena*, various circadian rhythms display temperature compensation [2], and we considered it unnecessary to examine this aspect for the Asc rhythm. The Asc rhythm also ran independently of cell cycle progression; this was particularly obvious in *Euglena*, and held true for spinach leaves that had finished their growth cycle. These findings support the ‘resistance to light hypothesis’ [7]. Furthermore, as the same rhythm was found in phylogenetically very distant species, we suggest that it is likely ubiquitous in photosynthetic organisms.

Defense against oxidative stress has been studied exclusively with respect to stress responses, and one of the convergent conclusions is that many, if not all, antioxidants (enzymes or non-enzymes) are activated in response to stress from various sources [18-20]. It must be emphasized that the circadian Asc rhythm reported here is not a stress response, but was generated by a circadian rhythm along a physiological time course, as though *Euglena* and spinach could anticipate, without an environmental time cue or stress, when oxidative stress would become most severe. The same was true for the circadian UV-C and UV-B-resistance rhythms in the alga [7].

4.1. Causes of the Asc rhythm

The changes in the Asc+DH-Asc level revealed two components. First, the level seemed to depend upon photosynthesis, at least in *Euglena*. Thus, it decreased considerably in DD, and the level in yellow LD: 1,1 was about half that in white LD: 1,1.
The spectral effect was not the result of altered activities of signaling pathways but rather altered photosynthetic activity (see Results). Photosynthetic capacity and activity under our culture conditions (constant light intensity) obeyed a circadian rhythm in-phase with the Asc rhythm [cf. 2]. As discussed below, the latter could not result from the former; the reverse may hold through the anti-oxidative protection of photosynthetic mechanisms. The second component is a circadian rhythm.

Interestingly, Shigeoka et al. [13] found that, in *E. gracilis* growing photoautotrophically and dividing synchronously under a 24-h LD cycle, both darkness and an inhibitor of photosynthesis applied before subjective midday were able to reduce the increase in the Asc+DH-Asc level during the light period. The overt manifestation of the Asc rhythm obviously depended on photosynthesis, most probably because *de novo* Asc synthesis is similarly dependent. Accordingly, our results revealed that the amplitude of the Asc rhythm strongly depended on lighting conditions.

Nevertheless, this does not mean that the driving force of the oscillation lies in photosynthesis; if Asc were depleted, we would not see its overt rhythm, even though the underlying (or driving) oscillation may persist. Indeed, circadian rhythms are able to persist in DD in *Euglena* [5, 7], as in many other organisms [1, 2]. This was also the case for the algal Asc rhythm, although its amplitude was considerably reduced in DD (Fig. 5). Similarly, the algal circadian rhythm of photo-induced cell division persists in DD, but light is absolutely required for the photo-induction [5].

The 90° (~6-h) lag of the Asc rhythm behind the rhythm of net synthesis in *Euglena* and net increase in spinach (Part B of Figs. 1-5, Fig. 6C) also indicates that the driving force of the Asc rhythm does not lie in photosynthesis. The causalities and phase differences that we have demonstrated show that photosynthesis cannot be the source of the Asc rhythm. The net increase in spinach may be also ascribable to net synthesis, although we cannot exclude the possibility that the Asc rhythm in the leaves results from the translocation between the leaves and the stems+roots; the measurement of Asc levels in whole plants will resolve this issue.

The net synthesis of Asc+DH-Asc is determined by the balance between synthesis and breakdown. The biosynthesis of Asc seems to be regulated at its final step in both mammals and plants [21]. This is likely true also for *E. gracilis*, because Shigeoka et al. [22] demonstrated that *de novo* synthesis of the terminal enzyme, L-gulono-γ-lactone dehydrogenase, is responsible for the increase in Asc+DH-Asc induced by the irradiation of dark-grown heterotrophic cultures of *E. gracilis*. Thus, we suggest that the enzyme activity may follow a circadian rhythm in phase with the circadian rhythm of the net synthesis of Asc. Alternatively, the breakdown of Asc+DH-Asc may be rhythmic, with
the constant synthesis, or both may display anti-phase rhythms. All these questions remain for future study.

4.2. Functions of Asc rhythms

Asc is a potent antioxidant [23, 24] with the ability to quench singlet molecular oxygen and scavenging radicals; the former activity is much stronger (Bolige and Goto, unpublished). Thus, the circadian Asc rhythm may contribute to maximizing the defense activity against photo-oxidative stress, which lends support to the ‘resistance to light’ hypothesis [7].

Asc also contributes to enzymic antioxidation as a substrate of ascorbate peroxidase, and thus it is part of the water-water cycle [25], scavenging of reactive oxygen species (ROS) generated through PET activity. As both solar radiation and PET capacity are highest at midday, so are PET activity and the generation of ROS derived from PET activity. Hence, the circadian Asc rhythm may be adaptive in this respect too. Although circadian rhythms of ascorbate peroxidase activity have not yet been reported in any organism, a circadian rhythm of superoxide dismutase, another enzyme of the water-water cycle, occurs in the dinoflagellate *Gonyaulax polyedra* [26].

We were most interested in the question of whether the Asc rhythm causes the UV-resistance rhythm, given that exogenously applied Asc greatly increases UV-C resistance, although it has a lesser impact on UV-B resistance in the alga [7]. The overall answer was negative, because the Asc rhythm rapidly waned in DD, quite in contrast to the robust persistence of the UV-resistance rhythm in DD [7]. This does not necessarily mean that the Asc rhythm plays no role in generating the UV-C resistance rhythm under active photosynthesis, when the Asc level was much higher than in DD. However, the alternative postulate, that the Asc rhythm may have nothing to do with UV-C resistance rhythm under any condition, is also tenable.

In either case, it is certain that, under DD, the Asc rhythm cannot be responsible for the UV-resistance rhythm and that we should seek circadian rhythms in levels of other antioxidants that persist vigorously in DD. They should be present, because UV-resistance involves non-enzymic antioxidants [7]. Moreover, our unpublished results (Bolige and Goto) revealed that, when extracts were prepared from *Euglena* at 2-h intervals in DD, the activities of both singlet molecular oxygen quenching and radical scavenging displayed robustly persistent circadian rhythms in-phase with the UV-resistance rhythm. Research into identifying the molecular species of these antioxidants is certainly warranted.
References


Figure legends
Fig. 1. Temporal changes in Asc and DH-Asc levels in *Euglena* about to enter stationary phase under LD: 12,12. (A) The intracellular levels of total ascorbate (Asc+DH-Asc) are designated by closed symbols, each of which represents an independent series of the measurement using the same culture, but from cells stocked separately and frozen. The open circles represent the 3-point moving averages of the means. Crossed circles represent the time series of the DH-Asc content. (B) The temporal change in the net increase rate (per 4 h) of total ascorbate. Symbols are as in (A).

Fig. 2. Temporal changes in Asc and DH-Asc levels in slowly dividing *Euglena* under white LD:1,1. Symbols are as in Fig. 1. (A) The intracellular level of Asc+DH-Asc and DH-Asc; (B) the net rate (per 4 h) of increase of Asc+DH-Asc; and (C) cell population growth.

Fig. 3. Temporal changes in Asc and DH-Asc levels in non-dividing *Euglena* under white LD:1,1. Symbols are as in Fig. 1. (A) The intracellular level of Asc+DH-Asc and DH-Asc; and (B) the net rate (per 4 h) of increase of Asc+DH-Asc.

Fig. 4. Temporal changes in Asc and DH-Asc levels in non-dividing *Euglena* under yellow LD:1,1. Symbols are as in Fig. 1. (A) The intracellular level of Asc+DH-Asc and DH-Asc; and (B) the net rate (per 4 h) of increase of Asc+DH-Asc.

Fig. 5. Temporal changes in Asc and DH-Asc levels in non-dividing *Euglena* under DD. (A) The intracellular level of Asc+DH-Asc and DH-Asc. The regression line denotes the linear trend of the change; and (B) The linear trend was subtracted from the original curve in (A), and the resultant time series was used to calculate the net rate (per 4 h) of increase of Asc+DH-Asc.

Fig. 6. Temporal changes in Asc level in spinach under LL. Closed and open circles represent the culture conditions LL at 30 klx/25°C and 20 klx/20°C, respectively. (A) The Asc content in the leaf blades. Vertical bars = SE. Solid and dashed straight lines represent the linear regression for the changes under 30 klx/25°C and 20 klx/20°C, respectively. (B) The linear trend was subtracted from the original curve in (A), and the resultant time series was smoothed using the 3-point moving average. (C) The net rate (per 4 h) of increase of the Asc content in leaf blades.
Figure 1

Ascorbate content (nmol/10⁶ cells)

Net increase of total ascorbate (nmol/4 h/10⁶ cells)

Time (h) in LD:12,12
Figure 2
Figure 3
Figure 4

Ascorbate Content (nmol/10^6 cells)

Net Ascorbate Increase (nmol/4 h/10^6 cells)

Time (h) in Yellow LD: 1,1
Figure 5
Figure 6

Figure 6 shows the ASC content (µmol/g FW) over time in LL conditions. The graph includes three panels:

A. ASC content (µmol/g FW)
B. ASC content (µmol/g FW) as the deviation from the linear trend
C. Net increase of ASC (µmol/g FW/4 h)

The x-axis represents time in LL (h), ranging from 0 to 72 hours, and the y-axis represents the ASC content or net increase values. The data points are represented by different symbols and error bars, indicating variability in the measurements.
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