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Involvement of Non-enzymatic Antioxidants in Circadian Rhythm Amplitudes of UV-C Resistance in *Euglena gracilis* Klebs

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ABSTRACT

Purpose : *Circadian rhythms of resistance to ultraviolet (UV) irradiation help prevent UV-induced damage during daytime hours in the photosynthetic organisms. The present study was performed to identify the primary causes of these rhythms using unicellular microalgal flagellate Euglena gracilis as a model.*

Research Method : *We examined the dose-response effects of the exogenous supplementation of 25 different antioxidants on enhancing resistance in E. gracilis to UV-C irradiation based on its immediate survival. We also compared radical scavenging and singlet-molecular-oxygen ($^1O_2$)-quenching activities of each antioxidant in homogeneous ethanol solutions.*

Findings : *Several antioxidants enhanced UV-C resistance at the least resistant phase to levels greater than or equal to levels achieved physiologically at the most resistant phase, suggesting that the antioxidants (or their physiological counterparts) may be under circadian control and may be responsible for generating amplitudes of the circadian UV-resistance rhythms. We also found that the antioxidants involved were different for UV-C resistance rhythms. However, no evident relationship between the effects of antioxidants on UV resistance and their antioxidative potential was observed.*

Originality / Value : *The study shows that the chemical properties of the antioxidants, rather than their general potencies, are responsible for generating the amplitudes of circadian UV-resistance rhythms.*

Keywords: *antioxidants, circadian rhythms, free radicals, photodamage, singlet oxygen*

INTRODUCTION

Photosynthetic organisms are faced with the dilemma of maximizing the capture of solar light for photosynthesis while risking photodamage. As an escape from photosynthesis-derived photodamage, sun-tolerant photosynthetic organisms, microalgae in particular, acclimate to bright irradiance by decreasing cellular chlorophyll levels and increasing non-photochemical quenching using xanthophyll cycles (Falkowski and Owens, 1980; Behrenfeld *et al.,* 2008); however, this is not an effective defense against UV irradiation. Motility and the surrounding habitat may help promote the localization of microalgae in areas of irradiance preference by phototaxis and photophobic responses (Clegg *et al.,* 2003). The optimal level of irradiance and, thus, the photosynthetic gain would be lower than the actual unless they are endowed with a mechanism referred to as circadian rhythms (Goto and Beneragama, 2010).

Circadian rhythms are biological rhythms that persist with a period of approximately 24 h under conditions with no time cues such as continuous light (LL) or darkness (DD) (Sweeney, 1987; Edmunds Jr, 1988). When microalgae are under

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periodic illumination, such as day/night or 24-h light/dark (LD) cycles, the rhythm is reset (or entrained) daily in order to establish a stable phase relationship with, and thus to anticipatorily adapt to the cycle. Circadian rhythms are widespread in organisms from cyanobacteria to humans and may play a significant role in gene expression, metabolism, physiology, morphology and behavior (Sweeney, 1987; Edmunds Jr, 1988). In the present study, the circadian rhythms related to UV-C resistance, which was first documented in the green alga *Chlamydomonas reinhardtii* (Nikaido and Johnson, 2000) and later in the flagellated alga *Euglena gracilis* (Bolige *et al.,* 2005), were examined. In both *C. reinhardtii* and *E. gracilis*, the maximum resistance occurs near subjective midday, an endogenous phase or state that arises at noon when the rhythm entrains to 24-h LD cycles. These rhythms persist under conditions in which cell-cycle dependent sensitivity to UV may be excluded and, therefore, may prepare the cell for midday (noon) when UV-stress is generally severe.

Viability (or immediate survival) and reproductive survival in *E. gracilis* after UV irradiation have been used as indices of UV resistance (Bolige *et al.,* 2005) and are regulated by circadian rhythms; however, the present study examined viability and biophysical/biochemical features that are associated with higher levels of UV resistance. Bolige *et al.,* (2005) reported that: (1) based on experiments conducted at 0 or 25 °C under UV irradiation, immediate survival does not involve an enzymatic defense; (2) damages to the membrane, through the oxidation of lipids and/or proteins, rather than to DNA kills the alga, because the viability was examined using Neutral Red (NR) that stains the protoplasm (or the cellular matrix) of UV-killed alga; (3) non-enzymatic antioxidants may be involved, because the viability was greatly enhanced by the exogenous antioxidants, L-ascorbic acid (Asc), and dimethylsulfoxide (DMSO). In addition, the intracellular level of Asc follows a circadian rhythm running in parallel with UV-C resistance rhythm but rapidly fades out in DD (Kiyota *et al.,* 2006) while the latter vigorously persists (Bolige *et al.,* 2005), suggesting that the Asc rhythm is not responsible for the rhythm of UV-C resistance at least in DD (Kiyota *et al.,* 2006). Bolige (2007) showed that the cellular resistance to both singlet molecular oxygen ($^1O_2$) and oxygen radicals follows a circadian rhythm that persists in DD, in accordance with circadian UV-resistance rhythms.

In the present study, we investigated the circadian rhythms of cellular resistance to antioxidants and $^1O_2$ or oxygen radicals and their possible association with UV-C resistance using 25 different antioxidants. We evaluated the effect of antioxidants on the UV resistance of *E. gracilis* at the least resistant phase. We reasoned that a positively effective antioxidant (or its physiological counterpart) may be responsible for increasing the resistance level in a circadian manner, i.e., enhancing the circadian amplitude (or different levels that continuously and rhythmically change according to the autonomous progression of the circadian phases) from basal levels, although these particular levels were not evaluated. Although many studies have examined antioxidants, little is known about their relative effectiveness on either quenching $^1O_2$ or scavenging oxygen radicals. Therefore, we characterized these in homogeneous solution and compared them with their potentials to enhance UV-C resistance from basal levels.

MATERIALS AND METHODS

Organisms and reagents

The algae, *Euglena gracilis* 'Z' were cultured axenically at 25 °C and photoautotrophically under LL with cool-white fluorescent lamps at 84 μmol m^{-2} s^{-1} (6 klx) according to Beneragama and Goto (2010) with the lamp irradiance spectrum as detailed in Bolige and Goto (2007). Approximately 8 ml of Euglena culture were withdrawn automatically every 2 h and fixed with 0.5 ml of 20% neutral formalin containing 20% NaCl. The cell number was counted with an electronic particle counter (Coulter Electronics, Inc., Hileah, FL, USA). When the cell titer reached 6 - 7 × 10^4 cells/ml, the algae were transferred to DD to arrest the cell-cycle progression (Hagiwara *et al.,* 2001).

Commercially available, analytical-grade reagents were used throughout the study. β-carotene, L-cysteine monohydrochloride monohydrate (Cys), 1,4-diazabicyclo[2.2.2] octane (DABCO), dimethylsulfoxide (DMSO), 1,1-diphenyl-2-picrylhydrazyl radical (DPPH), L-histidine, imidazole, DL-α-lipoic acid, lycopene, melatonin, DL-methionine, quercetin dihydrate, Rose Bengal (RB), rutin, L-ascorbic acid (Asc), α-tocopherol acetate, 6-Hydroxy-2,5,7,8-tetramethyl-chroman-2-carboxylic acid (Trolox) were purchased from Wako Chemicals, Inc. (Tokyo) and NR from Merck Japan (Tokyo). *N*-Acetyl-L-cysteine (NAC), astaxanthin, deferoxamine mesylate, DL-dihydrolipoic acid, dimethyl sulfone (DMSO2), 1,3-diphenylisobenzofuran (DPBF), epicatechin, glutathione-reduced form (GSH), GSH-ethylester, taurine, coenzyme Q10 (Q10) were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Stock solutions were freshly prepared before experiments as follows: β-carotene, deferoxamine, melatonin, α-tocopherol acetate, epicatechin, rutin, Trolox and quercetine dihydrate in 20% DMSO; astaxanthin and lycopene in 40% DMSO; DL-α-lipoic acid and DL-dihydrolipoic acid in 50% ethanol; DPPH, DPBF and Q10 in 98% ethanol. The stock solutions of the remaining reagents were prepared in water. Stock solutions with DMSO and ethanol as solvents were prepared so that the final concentrations in the reaction mixtures were <0.1% and <1% respectively. These solvent concentrations had no effect on the UV resistance in Euglena.

Evaluation of antioxidative potential

Twenty-five antioxidants were spectrophotometrically evaluated for their potential to scavenge radicals and quench $^1O_2$. Radical scavenging activity was determined by the reactivity of each antioxidant with DPPH according to Blois (1958) with a minor modification. The final assay solution of 1 ml consisted of various antioxidant concentrations, 0.1 mM DPPH and 80% ethanol. The samples were mixed gently and incubated in the dark for 30 min at room temperature (~25 °C). The decrease in absorbance at 520 nm was measured against a control sample without antioxidants. The DPPH-radical scavenging activity was calculated as follows: DPPH-radical scavenging activity (%) = [(absorbance of control – absorbance of sample) / (absorbance of control)] ×100.

The $^1O_2$ quenching activity of each antioxidant was determined by inhibition of DPBF oxidation, which is specific for $^1O_2$ (Carloni *et al.,* 1993). Photoexcitation of Rose-bengal (RB) can oxidize DPBF, generating mostly $^1O_2$. The final assay solution of 1 ml contained various antioxidant concentrations, 0.2 mM DPBF, 80% ethanol, and 15 μM RB. After shaking gently, 0.1 ml from each sample was dispensed into a 96-well plate. In order to minimize blue-light-induced oxidation of DPBF (absorption peak = 419 nm), samples were placed in the dark and irradiated using monochromatic light (λ_0 = 560 nm; half-width = 10 nm) at ~7 μmol m^{-2} s^{-1} for 1 min to photoexcite RB (absorption peak = 559 nm). Oxidized DPBF loses its color; therefore, absorbance at 415 nm was measured using a microplate reader (Bio-Rad model 550, Richmond, CA), before and after irradiation. The $^1O_2$ quenching activity was calculated as follows: $^1O_2$ quenching activity (%) = $[1 - (A_0 - A_1) / (C_0 - C_1)] \times 100$, where A_0 is the absorbance with the antioxidant before irradiation, A_1 is the absorbance with the antioxidant after irradiation, C_0 is the absorbance of the control before irradiation, and C_1 is the absorbance of control after irradiation.

Enhancement of UV-C resistance in the presence of antioxidants

Cell suspensions (5 ml) from cultures that had been transferred to DD were withdrawn every 2 h and placed in a Petri dish (3.7 mm diameter), which was then placed on a turntable automatically rotating at 15 rpm and was exposed to UV-C at various doses from above. UV-C radiation from a germicidal lamp (peak = 254 nm; GL-15, Panasonic, Tokyo, Japan) had an intensity of 2.2 W/m^2 to administer a dose of 1.1 kJ/m^2. UV-C doses were administered at the median lethal dose (LD_{50}) as previously determined (unpublished data). Immediately after exposure to UV irradiation, viable cells were

counted after staining with 0.03% NR. Cells with both cytoplasm and chloroplasts stained red and brown-red, respectively, were considered dead, and cells having a clear, unstained cytoplasm and chloroplasts with few or no red particles were considered alive.

The effect of exogenous supplementation of antioxidants on UV resistance was examined at the least resistant phase (44th h in DD) using UV-C dose of 1.1 kJ/m^2. Various antioxidant concentrations were added to the cell suspension and left for ~5 min before UV irradiation, and viable cells were counted by NR staining immediately after UV irradiation.

RESULTS AND DISCUSSION

Circadian rhythms and strengths of UV resistance

UV resistance for microalgae may be required mostly at around noon due to higher levels of radiation. However, in the present study, the maxima of *E. gracilis* Z did not occur at subjective midday, but rather at subjective dusk, although a circadian rhythm was observed (Fig. 1). The circadian waveform behaved as a relaxation oscillator instead of sinusoidally. The circadian minima of UV resistance occurring at subjective midday in the present study may completely contradict the 'resistance to light' hypothesis (Bolige *et al.,* 2005). However, the maxima that occur at noon may be required only for photoautotrophs with a weak UV resistance. In other words, photoautotrophs with a strong resistance to UV irradiation may not require a phase maximum at noon (Beneragama *et al.,* 2014).

EC_{50} values of antioxidants

We examined dose-dependent effects of antioxidants on the increase of UV-C resistance from basal levels in *E. gracilis* Z at the least resistant phase. We also studied the anti-DPPH (radical scavenging) and anti-"DPBF-oxidation" ($^1O_2$ quenching) activities of these antioxidants. Based on the dose-response curves, we determined antioxidant concentrations resulting in the half-maximal effect (EC_{50}) after UV-C irradiation and for radical scavenging and $^1O_2$ quenching activities. As shown in Table 1, the majority of the antioxidants showed similar orders of magnitude in EC_{50} values for both antioxidative activities and UV resistance. These results strongly suggested an increase in UV resistance due to the antioxidative activities, but not due to the side-effects of antioxidants.

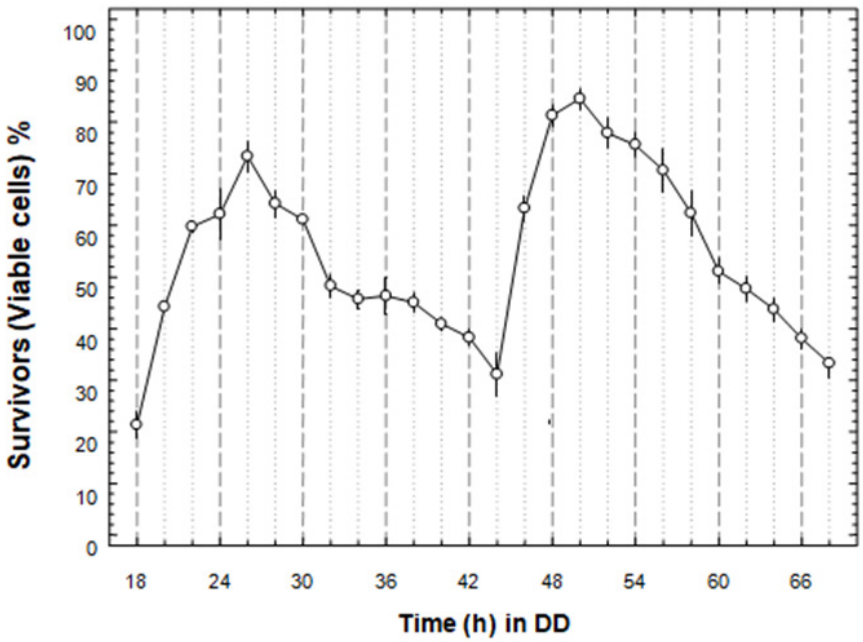

Figure 01: Circadian rhythms of survival after UV-C irradiation of Euglena cultures transferred from LL to DD. The UV-C dose was 19 kJ/m2.

Table 01: Effect of antioxidants on anti-DPPH, anti-$^1O_2$, and UV-C resistance. Dose responses were derived for antioxidants based on radical scavenging (anti-DPPH) and $^1O_2$ quenching (anti-DPBF oxidation) activities, and the increase in survival after UV-C irradiation. The concentration that resulted in a half-maximal effect (EC_{50}) and maximum survival (%) are presented.

			Hydrophilic antioxidants								Lipophilic antioxidants								Amphiphilic antioxidants				
			Ascorbic acid	Cysteine	GSH	GSH-Ethylester	Histidine	Methionine	NAC	Taurine	Alpha Tocopherol	Astaxanthin	Beta carotene	CoenzymeQ10	Deferoxamine	Lycopene	Quercetin	Rutin	DMSO	DMSO2	Epicatechin	Melatonin	Trolox
anti-DPPH	EC50	(M)	2.2	2.5	2.9	2	7.2	1.7	7	2.6	1.4	2.4	9.1	1.4	1.9	2.2	1.5	2.6	6.4	9.1	6.5	2.1	4.9
		a	-3	-3	-3	-3	-3	-4	-4	-3	-5	-5	-6	-5	-5	-5	-5	-5	-2	-2	-6	-4	-5
	Max. Activity	(%)	66.5	98.8	98.2	100	1.4	0.8	98.3	11.3	6.6	12	17.4	7.1	69.7	11.2	90.7	86.8	10.6	13.4	94.1	3.2	95.7
anti-DPBF-oxidation	EC50	(M)	4	3.5	1.3	7.3	9.5	1.2	6.8	3.3	1.4	3.6	9.6	-	7.2	1.1	3.2	1	3.8	1.8	3.8	9.3	2.6
		a	-4	-4	-3	-4	-4	-4	-4	-3	-4	-5	-6	-	-6	-5	-6	-5	-2	-1	-6	-5	-6
	Max. Activity	(%)	60.7	85.3	42.5	53	58.3	48.7	58.7	20.7	7.1	28.3	17.2	0	41.9	9.9	36.3	35	24.9	1.8	41.9	36.7	31.2
UV-C	EC50	(M)	3	3.1	3.1	2.3	8.7	3.6	4.7	4.2	-	2.2	1.7	6.9	7	1.7	-	5.4	1.2	-	7.8	1..3	5.3
		a	-4	-3	-3	-4	-3	-4	-3	-3	-	-5	-5	-5	-6	-5	-	-5	-1	-	-6	-4	-5
	Max. survival	(%)	80.9	62.2	72.8	55.9	77.2	37.4	59.9	80.5	27.5	34.5	43.4	62.6	39.9	35.6	30.2	56.1	52.6	28.3	37.4	80.2	39.7

[a] *order of molar concentrations.*

Association between circadian UV resistance and anti-reactive oxygen species (ROS)

Kiyota *et al.,* (2006) observed a circadian rhythm of intracellular Asc levels in Euglena that ran parallel to the UV resistance rhythm, suggesting that the former may be associated with the latter. However, this causal relation may be sustained only when the alga is irradiated, as only the UV resistance rhythm vigorously persisted in DD (Beneragama *et al.,* 2019). We examined antioxidant effects, which were supplemented exogenously at the least resistant phase (subjective midday, 44th h in DD), on the enhancement of UV resistance. The maximum antioxidant effects on the survival of *E. gracilis* after UV-C irradiation are shown in Table 1 and are plotted in the order of magnitude (Fig. 2A and 2B).

Some antioxidants increased both UV-C resistance from the basal levels, which were achieved at the least resistant phase, to levels of resistance equal to or greater than levels achieved physiologically at the most resistant phase. These antioxidants included Asc, melatonin, L-histidine and GSH. Taurine affected only UV-C resistance. Therefore, we suggest that circadian UV-resistance rhythms may result from circadian rhythms of antioxidant levels, although the specific antioxidants (or physiological counterparts) involved remain unknown. However, they may be implicated differently in UV-C resistance rhythms due to an overall order of effectiveness that differed remarkably for UV-C (Fig. 2A), suggesting differences in antioxidants responsible for generating the amplitudes of circadian rhythms of UV-C resistance, as previously mentioned by Bolige *et al.,* (2005).

Several antioxidants were categorized as completely ineffective (open circles in Fig. 2B) such as DABCO, DL-α-lipoic acid, DL-dihydrolipoic acid, imidazole, quercetin, and α-tocopherol. In addition, β-carotene, astaxanthin, lycopene, and epicatechin only slightly increased UV-C resistance above the minimum basal level. The ineffectiveness at the least resistant phase does not necessarily mean that these antioxidants may not be involved in UV resistance at all. For example, some antioxidants may determine the minimum level of the circadian rhythms, or basal level (at the least resistant phase), but may not be directly involved in generating the circadian amplitude.

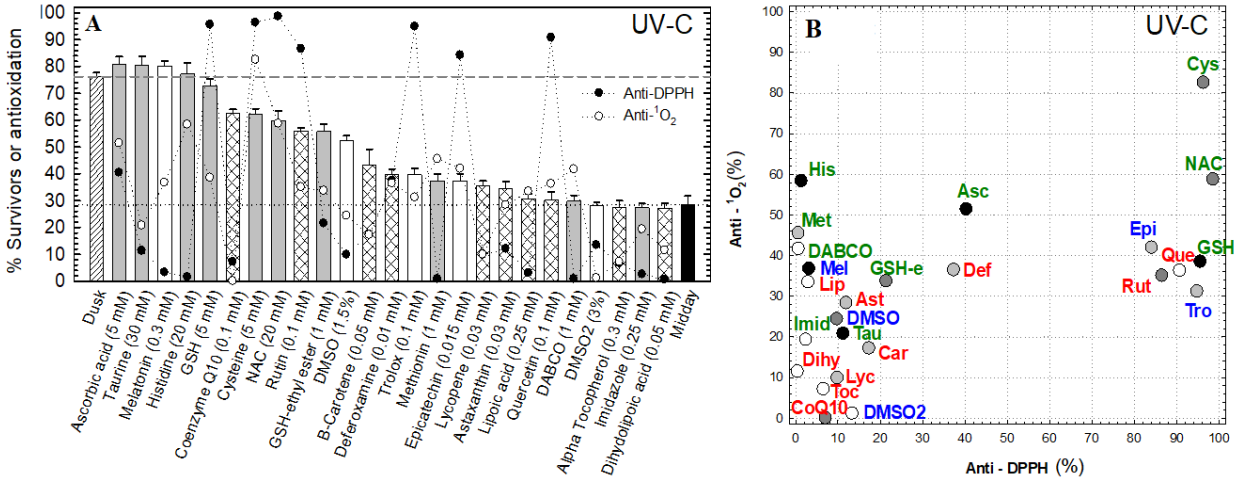

Figure 02: **Relative effectiveness of antioxidants for the survival after UV-C irradiation, and their anti-ROS activities. (A) Survival (%) after UV-C irradiation in the presence or absence of antioxidants. The horizontal lines represent the highest and lowest percentage survival of the controls, respectively, connecting the leftmost vertical hatched bar at the 50th h (subjective dusk) in DD and the rightmost closed bar at the 44th h (subjective midday). Grey, cross-hatched, and open bars represent hydrophilic, lipophilic, and amphiphilic antioxidants, respectively. Vertical lines above each bar represent the SEM. (B) Antioxidants were positioned according to their combined activities of radical scavenging and $^1O_2$ quenching. Redrawn from (A), antioxidants were categorized into 4 levels of effectiveness based on percentage survival: high effectiveness (black circles, >70%), moderate (dark grey, 50–70%), slight (grey, 35–50%) and ineffective (white, <35%). The text color represents the antioxidant solubility: green, hydrophilic; red, lipophilic; blue, amphiphilic.**

Therefore, it is not surprising to observe the ineffectiveness of α-tocopherol and carotenoids (e.g., astaxanthine, β-carotene and lycopene) in enhancing UV resistance at the least resistant phase, although they are well known for their high amounts and antioxidative activities (Blokhina *et al.,* 2003; Edge *et al.,* 1997; Fuchs, 1998; Lin *et al.,* 2002) especially α-tocopherol and β-carotene (Takeyama *et al.,* 1997; Fujita *et al.,* 2008). Likewise, the effective antioxidants found in this study, or their physiological counterparts, may not be associated with determining the circadian basal level, which is beyond the focus of this study, but rather with generating the circadian amplitude.

UV resistance versus antioxidant activities

In order to see whether the antioxidant effectiveness in generating circadian amplitudes of UV resistance rhythms described above is associated with the general antioxidant potency, we examined radical scavenging and $^1O_2$ quenching activities of the 'optimal antioxidant concentration,' i.e., the minimum concentration resulting in the highest cell survival after UV exposure (Fig. 2A). Using these data, we also plotted the antioxidant effectiveness on UV-C (Fig. 2B) resistance against the 2-D array of radical scavenging and $^1O_2$ quenching activities.

First five of the best antioxidants (Asc, taurine, melatonin, L-histidine, and GSH) that enhanced UV-C survival from basal levels showed relatively high $^1O_2$ quenching activities with variable radical-scavenging activities, except for the relatively low activity of taurine (Figs. 2A and 2B). Although these results suggested the involvement of $^1O_2$ quenching, other antioxidants with relatively high $^1O_2$ quenching activities, such as DABCO, DL-α-lipoic acid and quercetin, did not efficiently enhance UV-C survival.

Table 02: Multivariate linear regression analysis of dependence of UV-C resistance on anti-DPPH and anti-$^1O_2$ activities. Hydrophilic antioxidants include ascorbic acid, cysteine, GSH, GSH-ethylester, histidine, methionine, NAC, and taurine. Lipophilic antioxidants include astaxanthin, β-carotene, coenzyme Q10, deferoxamine, lycopene, quercetin, and rutin. Amphiphilic antioxidants include DMSO, DMSO2, epicatechin, melatonin and Trolox.

	coefficients		n	R^2	*P*
	DPBF	DPPH			
All antioxidants	0.47	-0.1	20	0.24	0.21
Hydrophilic antioxidants	-0.26	0.02	8	0.44	0.46
Lipophilic antioxidants	-0.45	0.15	7	0.51	0.48
Amphiphilic antioxidants	1.1	-0.41	5	0.91	0.38

On the other hand, of the seven antioxidants with the highest activities of radical scavenging, GSH was characterized as the best antioxidant for enhancing UV-C survival from basal levels, whereas Cys, NAC, and rutin ranked second best. Trolox, epicatechin, and quercetin had little or no effect on UV-C survival levels at the least resistant phase, suggesting a lack of entry into the algal cells (Fig. 2B). It should also be noted that taurine and Q10 were relatively highly effective in enhancing UV-C survival, but have relatively low antioxidative activities. Collectively, these findings indicate that the correlation between effectiveness in enhancing UV-C resistance and antioxidative activity appears to be minimal when assessed according to the methods used in this study.

The multivariate linear regression analysis supported the notion that the antioxidant effects on enhancing UV resistance from basal levels cannot be explained by their general potencies, which were assessed by radical scavenging or $^1O_2$ quenching activities ($p > 0.05$) in homogeneous ethanol solutions (Table 2). In the regression analysis, we excluded antioxidants (imidazole, lipoic acid, dihydrolipoic acid, α-tocopherol, and DABCO) that had no effect on UV-C survival, which did not alter our general conclusion. Based on the statistical analysis, the increase in UV resistance did not depend on these activities, irrespective of their chemical properties (hydrophilic, lipophilic, and amphiphilic). It becomes clear that the general potency of antioxidation as assessed in the homogeneous solution may not be involved in generating the circadian amplitudes, but this does not necessarily mean that the antioxidants (or their physiological counterparts) may generate the amplitudes through their anti-ROS activities, which may require some chemical features of the antioxidants. A better technique, such as microinjection might render clearer results.

CONCLUSION AND FUTURE PROSPECTS

We confirmed that the circadian rhythms of antioxidant levels may underlie those of UV-C resistance, as reported by Bolige *et al.,* (2005). We showed that several antioxidants enhanced either UV-C resistance from basal levels at the least resistance phase to or greater than the physiological level of resistance achieved at the most resistant phase. It should be noted that these antioxidants (or their physiological counterparts) may be responsible for generating the amplitudes of the circadian UV-resistance rhythms. However, these results did not clarify if they are involved in constituting the basal levels of the resistance rhythms. Moreover, we confirmed that the antioxidants involved in the UV-C resistance rhythms should be different from one another.

Bolige *et al.,* (2005) ascribed the circadian UV-B resistance to the circadian rhythms of anti-hydroxylradical levels, but our results suggest differently. In the present study, we used 25 antioxidants, whereas Bolige *et al.,* (2005) supplemented only with Asc and DMSO. We found no apparent relationship between UV resistance and the general potency of radical-

scavenging or $^1O_2$-quenching activities of these antioxidants.

Further studies should also consider evaluating the particular antioxidants that are physiologically involved in enhancing the circadian amplitudes of UV resistance rhythms. Finally, the present study was not intended to discern the compounds responsible for constituting (or maintaining) basal levels of circadian UV resistance at the least resistant phase; however, our results suggest that α-tocopherol and β-carotene might be potential candidates.

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