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Co-stressors chilling and high light increase photooxidative stress in diuron-treated red alga *Kappaphycus alvarezii* but with lower involvement of H_2O_2

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ABSTRACT

Diuron is one of the most commonly found *N*-phenylurea herbicides in marine/estuarine waters that promotes toxic effects by inhibiting photosynthesis and affecting the production of reactive oxygen species (ROS) in autotrophs. Since photo- and thermoacclimation are also ROS-mediated processes, this work evaluates a hypothetical additive effect of high light (HL) and chilling (12 °C) on 50 nM diuron toxicity to the highly-photosynthetically active apices of the red alga *Kappaphycus alvarezii*. Additive inhibition of photosynthesis was mainly evidenced by significant decreases of quantum yield of photosystem II and electron transfer rates upon co-stressors exposure to diuron-treated algae. Under extreme 12 °C/HL/diuron conditions, unexpected lower correlations between H_2O_2 concentrations in seawater and radical-sensitive protein thiols were concomitantly measured with the highest indexes of photoinhibition (parameter β). Altogether, these data support the hypothesis that co-stressors chilling/HL additively inhibit photosynthesis in diuron-exposed *K. alvarezii* but with less involvement of H_2O_2 in injury effects than with only chilling or HL.

1. Introduction

The *N*-phenylurea herbicide diuron (or DCMU; 3-(3,4-dichlorophenyl)-1,1-dimethylurea) is extensively used in sugarcane, cotton, coffee, citrus and grape cultivation for pre-emergence weed control, and as a consequence, is a common contaminant of cultivated land soils. Studies on agrochemical dispersion have shown that diuron enters adjacent freshwater ecosystems from agricultural fields, making their way to estuarine areas by spray drift, leaching, run-off or accidental spills [1]. Indeed, this herbicide is commonly found in coastal waters and sediments worldwide, and often is one of the most commonly found herbicides in marine and estuarine waters [2].

Several herbicides, including diuron, promote their toxic effects on photosynthetic organisms by affecting the production of reactive oxygen species (ROS) in chloroplasts, leading to severe changes in the redox state of the plastoquinone pool [3]. Photo- and thermoacclimation of algal species are also regulated by the reduced/oxidized ratio of plastoquinone pool at the thylakoid membrane [4], with pivotal participations of H_2O_2 [5–7] and ascorbate/glutathione pools [8]. Thus, it is reasonable that concomitant light and temperature stress can enhance ROS-mediated toxicity of some xenobiotics by an additive principle, although different ROS might be involved [9,10].

Chlorophyll fluorescence techniques have accurately contributed to the understanding on how anthropogenic xenobiotic stresses inhibit primary production and photosynthesis efficiency in plants and algae [11]. Rapid light curves (RLCs) analyze the fluorescence responses of plant/algal tissues to increasing photosynthetically active radiations (PAR) and are advantageous for better understanding the overall photosynthetic performance in autotrophs under natural or stress conditions [12]. Several mathematical equations have been postulated to compare photosynthesis activity and PAR in autotrophs [13]. Among them, the

Abbreviations: APX, ascorbate peroxidase; BHT, butylated hydroxytoluene; CAT, catalase; CD, conjugated dienes; CHL a, chlorophyll a; DCMU or diuron, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; DBMIB, 2,5-dibromo-3-methyl-6-isopropyl-p-benzoquinone; DTNB, 5,5′-dithio-*bis*(2-nitrobenzoic acid); F_o, basal fluorescence of a dark-acclimated alga; F_m, maximal fluorescence of a dark-acclimated alga; F_v, variable fluorescence; FW, fresh weight; GSH, reduced glutathione; HL, high light; LL, low light; NBT, nitroblue tetrazolium; PAR, Photosynthetically active radiation; PQ, plastoquinone; PS II, photosystem II; ETR, electron transfer rate; RLC, rapid light curves; RNS, reactive nitrogen species; ROS, reactive oxygen species; SOD, superoxide dismutase; TBARS, thiobarbituric acid-reactive substances; Y_{PSII}, quantum yield of photosystem II.

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photoinhibition model described by Platt et al. [14] with the modifications proposed by McBride [15] better fit experimental data indoors or in the field, mainly due to the inclusion of exponential factors (β and θ) that properly account for photoinhibition of O_2-evolution after the P_{max} threshold [16].

Thus, in order to check the hypothesis that concomitant disfavored light and temperature conditions can enhance ROS-mediated toxicity of herbicides, a complete set of chlorophyll fluorescence parameters from RLCs were here compared to indexes of ROS metabolism in the highly-photosynthetically active apices of the red alga *Kappaphycus alvarezii* (Doty) Doty ex Silva (Gigartinales: Rhodophyta) exposed to 50 nM diuron under regular (26 °C) or chilling (12 °C) conditions and/or low light (LL; 50 μmol photon m^{-2} s^{-1}) or high light intensities (HL; 500 μmol photon m^{-2} s^{-1}).

2. Material and methods

2.1. Chemicals

All the chemicals were purchased from Sigma–Aldrich (St. Louis, MO), except NaOH, HCl, 70% $HClO_4$ solution, ethanol, ethyl acetate, and 30% H_2O_2 solution, purchased from Merck (Darmstadt, Germany). Regular laboratory solutions and buffers were obtained from Labsynth (Diadema, SP, Brazil).

2.2. Algal cultures

Clones of *K. alvarezii* (Doty) Doty ex Silva originally from the Philippines, have been cultivated in marine field stations in Ubatuba (SP, Brazil) since 1995 [17]. Branches of *K. alvarezii* (1.5 kg) were cultured in the laboratory in sterile seawater Guillard f/2 medium [18], at (25 ± 2) °C under white fluorescent light (55 μmol photons m^{-2} s^{-1}), light:dark (L:D) cycle of 12:12 h, and constant supply (bubbling) of atmospheric air.

2.3. Light and temperature conditions

Apices of algal thalli (1.5 g FW in 100 mL medium) were placed in two temperature-controlled incubators (Nova Etica 411/D86) set to 26 °C for regular temperature or 12 °C to impose chilling stress. In order to provide low light (LL) or high light (HL) conditions, the experimental flasks were positioned at different distances from the apparatus' door lamps and neutral black meshes were used to allow fine-adjustment of light irradiance. The irradiance at flask surface was measured with a Li-Cor Li-189 quantameter with a spherical quantum sensor Li-193 SA. Average values for LL and HL intensities were (54.3 ± 4.8) and (505 ± 34) μmol photon m^{-2} s^{-1}, respectively. Noteworthy, the 26 °C/LL experimental conditions described herewith match those in which algal branches were kept in the laboratory (stocking conditions).

2.4. Diuron treatment

Based on our previous studies [4,19], a low single-concentration of 50 nM (11.7 μg L^{-1}) diuron was added to *K. alvarezii* cultures to impose significant 10–15% inhibition of algal photosynthesis (from a 10 μM stock solution in acetone). The applied herbicide dose is in the same range as observed in contaminated natural waters [20,21] or in other algal studies focused on photosynthesis inhibition [22].

2.5. Chlorophyll a fluorescence

Chlorophyll *a* fluorescence was measured using a Walz Diving-PAM underwater fluorometer (Walz, Effeltrich, Germany). Removed from experimental flasks at times 0, 2, 4, 8, 16 and 24 h after treatment, apices of algal thalli (1.5 g FW) were placed directly on the tip of the fluorometer fiberoptic and immobilized with the supplied magnet sample holder for measurements (triplicates). Rapid light curves (RLCs) were obtained from fluorescence responses to eight increasing photosynthetically active radiation (PAR) levels within the range of 0–690 μmol photon m^{-2} s^{-1} [23]. The exposure time at each PAR level was 15 s, each separated by a 0.8 s saturating flash (~8000 μmol photon m^{-2} s^{-1}).

The calculation of quantum yield of photosystem II (Y_{PSII}) at different light intensities allows the estimation of the PAR fraction directly apportioned to PSII and converted into electron flow in thylakoids, affording the fluorescence parameter described as relative electron transport rate (ETR) [24]. Fluorescence data for determination of ETR and Y_{PSII} were obtained at the fixed PAR of 191 μmol photon m^{-2} s^{-1} using the WinControl software supplied (equations as follows):

$$Y_{PSII} = (F\prime_m - F_o)/F\prime_m = F\prime_v/F\prime_m \quad (1)$$

$$ETR = Y_{PSII} \times PAR \times A \times 0.5 \quad (2)$$

where:

F_m is the maximal fluorescence of a dark-acclimated alga; F'_m is the maximal fluorescence of a light exposed alga; F_o is the basal fluorescence of a light exposed alga; F'_v is the variable fluorescence; A is the absorbance factor measured for each replica and 0.5 is the assumed proportion of photons absorbed by pigments associated with PSII [25].

The photosynthetic parameters α (photosynthetic efficiency), ETR_{max} (maximum electron transfer rate), and β (photoinhibition parameter) were obtained by Eq. (3) [14]. The PAR intensity above which saturation becomes dominant is called the 'saturation index', I_k (Eq. (4))[24]. On the other hand, the dimensionless parameter θ described by McBride [15] is conceived as the most appropriate index to evaluate photoinhibition since it aggregates both exponential decay constants α and β (from Platt's photoinhibition model) with the further advantage that θ does not vary with I_k and P_{max} (Eq. (5)).

$$ETR = ETR_{max}[1 - \exp(-\alpha PAR/ETR_{max})]\exp(-\beta PAR/ETR_{max}) \quad (3)$$

$$I_k = \alpha/ETR_{max} \quad (4)$$

$$\theta = \alpha/\beta \quad (5)$$

The mathematical model was simulated from standard initial ETR_{max}, α and β values of 25, 0.05 and −0.01, respectively [26]. The final values of α were actually determined by linear fitting of the first three lowest PAR irradiances of the RLCs [27].

2.6. Chlorophyll a and protein quantifications

Chlorophyll *a* contents were evaluated in 500 μL methanol-extracted samples by measuring the corrected absorbance at 665 nm (subtracting the absorbance measured at 750 nm), and using the mass extinction coefficient of $\varepsilon = 27.6$ μg chlorophyll a^{-1} cm^{-1} [28]. Protein concentrations were estimated using bovine serum albumin as standard [29].

2.7. H_2O_2 determinations

Concentrations of H_2O_2 in seawater were estimated in 1 mL samples from *K. alvarezii* cultures at time 0, 2, 3, 4.5, 6 and 7 h after treatments. In order to exclude previous H_2O_2 contamination in the medium, each experimental algal culture was treated with 0.05 U L^{-1} of the peroxide-removing enzyme catalase (100-fold lower than basal catalase activity in *K. alvarezii* tissues). Briefly, a stock solution of luminol (5-amino-2,3-dihydro-1,4-phthalazinedione)