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Herbicidal effects of harmaline from *Peganum harmala* on photosynthesis of *Chlorella pyrenoidosa*: Probed by chlorophyll fluorescence and thermoluminescence

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ABSTRACT

The herbicidal effects of harmaline extracted from *Peganum harmala* seed on cell growth and photosynthesis of green algae *Chlorella pyrenoidosa* were investigated using chlorophyll *a* fluorescence and thermoluminescence techniques. Exposure to harmaline inhibited cell growth, pigments contents and oxygen evolution of *C. pyrenoidosa*. Oxygen evolution was more sensitive to harmaline toxicity than cell growth or the whole photosystem II (PSII) activity, maybe it was the first target site of harmaline. The JIP-test parameters showed that harmaline inhibited the donor side of PSII. Harmaline decreased photochemical efficiency and electron transport flow of PSII but increased the energy dissipation. The charge recombination was also affected by harmaline. Amplitude of the fast phase decreased and the slow phase increased at the highest level of harmaline. Electron transfer from Q_A^- to Q_B was inhibited and backward electron transport flow from Q_A^- to oxygen evolution complex was enhanced at 10 μg mL^{-1} harmaline. Exposure to 10 μg mL^{-1} harmaline caused appearance of C band in thermoluminescence. Exposure to 5 μg mL^{-1} harmaline inhibited the formation of proton gradient. The highest concentration of harmaline treatment inhibited $S_3Q_B^-$ charge recombination but promoted formation of $Q_A^-Y_D^+$ charge pairs. *P. harmala* harmaline may be a promising herbicide because of its inhibition of cell growth, pigments synthesis, oxygen evolution and PSII activities.

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1. Introduction

Allelopathy exists widely in nature. Plant, extracts of plant or plant chemical compounds can produce direct or indirect effect on another plant or microbe by releasing phytotoxins [1,2]. Recently, as an environmentally safe method, allelopathic substances are often suggested as potential natural products to control algal or cyanobacterial blooms in lakes [3]. There is an increasing interest in identifying the allelopathically active macrophyte species as control measures for bloom-forming algae or separating potential natural phytotoxins as effective biological algaecides [4]. Many macrophyte species inhibit growth of cyanobacteria [4,5].

Most allelopathic compounds are more biodegradable and environmentally friendly than traditional herbicides. Some commercial herbicides, such as diuron, atrazine and hexazinone, are photosystem II (PSII) inhibitors by competing with plastoquinone at Q_B binding site of D1 protein in reaction center and inhibiting energy transfer while other herbicides, such as paraquat, act on photosystem I (PSI) [6]. Phytotoxins can produce inhibitory effects as commercial herbicides but may act on different target sites on other plants or algae. Some phytotoxins could influence the growth of neighbor plants or algae [3,7] and change their respiration, photosynthesis, membrane permeability, cell division and development, protein synthesis and enzyme activity [8]. Some phytotoxins directly inhibit photosystem II (PSII) activity, dark respiration and ATP synthesis and induce damage to the antioxidant system [9–11], or inhibit photosynthetic oxygen evolution of intact cyanobacteria, or shift the maximum temperature of the B-band ($S_2Q_B^-$ recombination) to higher temperatures [9], or inhibit the electron transport [11,12].

Peganum species distribute wildly in Africa, Middle East, central Asia, South America, Mexico and southern USA [13]. More specifically, the medicinal herb *Peganum harmala* L. is distributed

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throughout northwest China [14] and is used to treat some diseases [15]. Extract of *P. harmala* is phytotoxic, decreases seed germination [16] and inhibits growth of neighboring plants [14]. Alkaloids, such as harmaline, harmine and harmalol, are the main chemical ingredients extracted from *P. harmala*, which are responsible for the antimicrobial, antidepressant and analgesic activities of *P. harmala* [14,17]. Dicot seedling growth is inhibited by 5 $\mu g\ mL^{-1}$ harmaline [14]. However, mechanisms of herbicidal effects of harmaline on the physiological processes are still not fully understood. To better understand the effects of harmaline on photosynthesis, its effects on cell growth, electron transport and charge recombination in PSII of the model green algae, *Chlorella pyrenoidosa*, were investigated using *in vivo* chlorophyll fluorescence and thermoluminescence techniques.

Chlorophyll (Chl) *a* fluorescence technique is a sensitive and reliable method to examine the action mode of herbicide on photosynthesis of algae *in vivo* [6,18]. Upon exposure to a strong actinic light, the increase in Chl *a* fluorescence intensity of dark-adapted photosynthetic materials will follow a triphasic kinetic curve starting from its initial level (F_o) , two intermediate level (F_J and F_I) and to its maximal level (F_M or F_P) [19] (called as OJIP). This OJIP curve reflects the successive but overlapping filling-up of photosystem II electron acceptor pools as Q_A, Q_B and PQ, whose redox states are controlled by PSII functions [19]. It can be used to analyze changes in electron transfer reaction on both donor and acceptor sides of PSII [20,21]. Based on the OJIP transients, Strasser and his team developed the "JIP" test. The JIP test translates the original data to biophysical parameters that can quantify the energy flow through the reaction center of PSII [22]. The decay of chlorophyll fluorescence (the relaxation kinetics of flash-induced fluorescence) specially provides information of the reoxidation of Q_A^- via forward electron transport to Q_B and back reactions with the S_2 state of the oxygen evolving complex [23,24]. Thermoluminescence (TL) is another particular technique to study luminescence, which can reveal different types of charge pairs and subtle changes in charge recombination events in PSII photochemistry [25–27]. In the present study, tests of Chl *a* fluorescence rise transient and relaxation kinetics of flash-induced fluorescence and thermoluminescence techniques were used to probe the targeted action sites of harmaline on electron transport chain in PSII of *C. pyrenoidosa*.

2. Materials and methods

2.1. Extraction and isolation of harmaline

Three hundred grams of *P. harmaline* seeds were ground into powder and extracted with 500 mL of 80% ethanol [14]. The ethanol extract was concentrated under reduced pressure to yield 52 g of dark red residue which was subsequently dissolved in 200 mL of 5% HCl and filtered. The filtrate was then partitioned three times with 200 mL of chloroform; the chloroform extracts were combined and dried under reduced pressure to give 250 mg of substances, which did not show any major spots on a TLC plate and was then discarded. The aqueous acid layer was made alkaline to pH 9 with NH_4OH and extracted four times with 200 mL of chloroform to yield 8 g of dry chloroform extract. The chloroform extract was then recrystallized in ethanol to give 3.5 g of yellowish crude crystals of total alkaloids. The crystals were further separated on a silica gel column eluted with a step gradient elution (EtOAc/MeOH at 1:0, 98:2, 96:4, 9:1, 8:2, 7:3, 6:4, 1:1, 0:1). Fractions were collected based on TLC profiles and purified to give 140 mg harmaline (detailed method refers to Shao et al. [14]). Structures of compounds were identified by comparing spectral data with published literature [14]. The purified harmaline was used for phytotoxicity test. Harmaline was dissolved in DMSO and diluted to the desired concentrations. 0.1 mL of deionized water or various concentrations of harmaline was added into 20 mL of cell suspension samples to obtain the final nominal harmaline concentrations of 0, 0.5, 1, 5 and 10 $\mu g\ mL^{-1}$. The same volume of harmaline-free DMSO solution was used as control.

2.2. Culture of C. pyrenoidosa

C. pyrenoidosa (FACHB-9) was obtained from the Institute of Hydrobiology, Chinese Academy Sciences. The algae cells were cultivated in BG-11 medium at 25 °C under 30 μmol photons $m^{-2}\ s^{-1}$ illumination with a 12/12 h light/dark period. Cells in the exponential growth phase were transferred into 50 mL conical flasks for toxicity experiments.

2.3. Measurement of cell growth

After exposure to harmaline for 0, 24, 48, and 72 h, the optical density at 680 (OD_{680}) of *C. pyrenoidosa* cultures was measured with a UV–vis spectrophotometer (UV2800, Unico, Shanghai, China).

The inhibitory effect (IC_{50}) of 72 h treatment of harmaline on the algal growth was estimated from the data that shows a 50% reduction in cell growth of harmaline treated samples compared to control.

2.4. Measurement of pigments content

72 h after exposure to harmaline, *C. pyrenoidosa* cells were harvested by centrifugation at 8000 r min^{-1} at 4 °C for 5 min and the pigments were extracted with 96% ethanol for 24 h at 4 °C in the dark followed by centrifugation (8000 r min^{-1}, 4 °C, 5 min). Absorption at 470, 649 and 665 nm of the supernatant was measured with the spectrophotometer (UV2800, Unico, Shanghai, China). Chl *a*, Chl *b* and carotenoids contents were calculated [28].

2.5. Measurement of oxygen evolution

Oxygen evolution was measured with a Clark-type oxygen electrode (Oxygraph, Hansatech Instruments Ltd., King's Lynn, Norfolk, England). 2 mL of algal cell suspension was added into the reaction cuvette for 5 min oxygen evolution. A white light with illumination on the surface of the cuvette of 150 μmol photons $m^{-2}\ s^{-1}$ were continuously provided during measurement of O_2 evolution (nmol $mL^{-1}\ min^{-1}$) [29].

2.6. Measurement of polyphasic fast fluorescence induction and calculation of JIP test

The polyphasic fast fluorescence induction was performed by using a double-modulation fluorometer (FL3500, PSI, Brno, Czech). The cells used for Chl *a* fluorescence measurements were dark-adapted for 30 min before each test. The polyphasic fluorescence transient was measured with a 1 s multiple turnover flash and recorded every 10 μs for the first 2 ms and every 1 ms up to 1 s [30].

The JIP-test was calculated to analyze each of Chl *a* fluorescence transient [22]. F_o (fluorescence intensity at 50 μs), F_J (fluorescence intensity at around 2 ms) and F_M (maximal fluorescence intensity, usually reached at 200–500 ms) from the original measurements were used. $F_{300\ \mu s}$ are required for calculation of the initial slope (M_o) of the relative variable fluorescence (V) kinetics. Some selected JIP-test parameters were derived from the fluorescence induction kinetics according to Strasser et al. [31]: V_j, relative variable fluorescence at J level; M_o, the initial slope, indicating the net

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