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Effects of Artemisinin on Photosystem II Performance of *Microcystis aeruginosa* by In Vivo Chlorophyll Fluorescence

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Abstract Effects of artemisinin (derived from *Artemisia annua*) on the photosynthetic activity of *Microcystis aeruginosa* was investigated by using chlorophyll *a* (Chl *a*) fluorescence transient O-J-I–P and JIP-test after exposure to elevated artemisinin concentration. High artemisinin concentration resulted in a significant suppression in photosynthesis and respiration. Results showed that the OJIP curves flattened and the maximal fluorescence yield reached at the J step under artemisinin stress. The decreased values of the energy needed for the RCs' closure (Sm) and the number of oxidation and reduction (N) suggested that the reduction times of primary bound plastoquinone (Q_A) was also decreased. The absorption flux (ABS/RC) per photosystem II (PSII) reaction center and the electron transport flux (ET_0/RC) decreased with increasing artemisinin concentration. Excess artemisinin had little effect on the trapping flux (TR_0/RC). The results showed that the decrease of photosynthesis in exposure to excess artemisinin may be a result of the inactivation of PSII reaction centers and the inhibition of electron transport in the acceptor side.

Keywords Artemisinin · *Microcystis aeruginosa* · Photosystem II · Chlorophyll fluorescence

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A new anti-algal substance artemisinin isolated from compositae plant *Artemisia annua* has been confirmed to possess strong inhibition activity on *Microcystis aeruginosa* (*M. aeruginosa*) (Ni et al. 2012). Artemisinin inhibits protein synthesis and induces increase in ROS level in algal cells (Ni et al. 2012). Cyanobacteria are photoautotrophic prokaryotes that are important plankton organisms in the sea and freshwater lakes (Srivastava et al. 1998). It has been shown that cyanobacterin from *Cyanobacterium scytonema hofimanni* inhibits electron transport in photosystem (Carlson et al. 1987; Lee and Gleason 1994). A significant inhibition of photosynthesis by high artemisinin concentration appears to be associated with the photosystem II (PSII), however, the mechanism behind is not understood clearly. Little information is available on the effect especially during the initial stages of the response to artemisinin stress.

Chlorophyll fluorescence has been proved to be a powerful, non-invasive and reliable tool for the study of the behavior of PSII (Krupa et al. 1993; Srivastava et al. 1998; Xia et al. 2004). Fast Chl *a* fluorescence induction kinetics provides information on the filling-up of the plastoquinone (PQ) pool which is affected by both the electron donor and acceptor sites of PSII (Hsu 1993; Shinkarev and Govindjee 1993; Govindjee 1995; Strasser et al. 1995). The maximal rate of photochemical reaction, when all reaction centers (RCs) are open, can be determined precisely by measuring the initial slope. When illuminated with high intensity actinic light, dark-adapted oxygenic photosynthetic

organisms show a polyphasic rise (also known as OJIP rise) with the basic steps from the "origin" (O) through two "inflections" (J and I) to a "peak" fluorescence level (P) (Strasser et al. 1995). The polyphasic fast-phase fluorescence induction curve provides valuable information on the magnitude of stress effects on PSII function. Moreover, those changes in PSII photochemistry can be quantified through the JIP test, which is derived from the O-J-I–P rise of chlorophyll fluorescence transients based on the theory of energy flux in biomembrances (Strasser 1978). Environmental stresses such as high temperature (Strasser 1997), light (Lu and Vonshak 1999), salinity (Xia et al. 2004), and heavy metal (such as Cr) inducing the change of OJIP chlorophyll fluorescence transients have been tested (Appenroth et al. 2001). The aim of the current study was to investigate the 72 h effects of artemisinin stress on PSII by analyzing the polyphasic rise of chlorophyll *a* fluorescence transients (O-J-I–P), and to determine the target sites of PSII under different concentrations of artemisinin in *M. aeruginosa*.

Materials and Methods

The strain of *M. aeruginosa* obtained from FACHB (Freshwater Algae Culture Collection of the institute of Hydrobiology, China) was pre-cultured in sterilized BG11 medium at 25°C under $40 - 60$ μmol photos m^{-2} s^{-1} (14 h light/10 h dark) conditions. Cultivation was performed in batches under exponential growth conditions. Artemisinin (>99 % pure), known as qinghaosu, was purchased from Nanjing Zelang Medical Technology Co., LTD, China. Artemisinin was dissolved in dimethylsulfoxide and stored at 4°C until use.

The algal cells at exponential growth phase were harvested and diluted to 1×10^6 cells mL^{-1} and used as test samples. According to EC_{50} values and physiological effects of artemisinin on *M. aeruginosa* (Ni et al. 2012), the initial concentration of artemisinin added were determined as 0, 4, 8, 12, and 20 mg L^{-1} to investigate the effects on the photosynthetic activity. The sample with 0 mg L^{-1} was used as control. All the samples were cultured under the same conditions described above. The chlorophyll a fluorescence was measured every day. Chlorophyll fluorescence transients were measured at room temperature with Fluropen (FP 100, Czech Republic). All samples were dark-adapted for 15 min before measurements. Light was provided by an LED array (650 nm) focused onto the sample to provide homogeneous irradiance over the exposed area (d = 4 mm). The fluorescence signals were detected by a high performance PIN photodiode detector with 697 to 750 nm bandpass filters and recorded within a time span of 10 μs to 3 s. Chlorophyll fluorescence transient was analyzed using the JIP-test developed by Strasser and Srivastava (Strasser et al. 1995; Strasser et al. 2000). The fluorescence intensities at 50 μs, 2 ms (J-step), 30 ms (I-step) and the maximal fluorescence (P-step) were denoted as F_O, F_J, F_I, and F_P respectively. Selected JIP-test parameters quantifying PSII behavior were calculated from the above original data and the formulae listed in Table 1.

The experiments were analyzed using a one-way ANOVA test ($p < 0.05$) using the SPSS 17.0 software.

Results and Discussion

Effects of artemisinin on the function of PSII of *M. aeruginosa* using fast chlorophyll fluorescence induction test were investigated. Fig. 1 shows the fast chlorophyll fluorescence curves of *M. aeruginosa* cells treated with different concentrations of artemisinin after 72 h. As expected, the peak of the maximum fluorescence intensity F_m (P level) disappeared and the J-I–P phase gradually leveled off with increasing artemisinin concentration. Only the blanks and 4 mg L^{-1} artemisinin concentration showed OJIP curve characteristics, the rest of the treatment groups just had O-J and J-I–P stages before leveling off.

The O-J-I–P transients reflected successive reduction of the electron acceptor pools of PSII (Govindjee 1995). Both experimental results and theoretical simulations also showed that J step represented an accumulation of $Q_A^- Q_B$ form (Govindjee 1995). Thus, the I step is thought to reflect an accumulation of the $Q_A^- Q_B^-$ form, whereas the P step an accumulation of $Q_A^- Q_B^{2-}$ form (Strasser et al. 1995; Lazar 1999). In the present study, artemisinin stress resulted in a sharp change in the fluorescence transients. Addition of artemisinin led to a transformation of the O-J-I–P rise into an O-J rise and the maximal fluorescence yield reached at the J step, which suggested that the artemisinin stress blocked the electron transfer from the primary bound plastoquinone Q_A^- to the secondary bound plastoquinone Q_B.

As the O-J-I–P fluorescence transient reflects the state of Q_A, Q_B and PQ pool, more information about the fluxes of photons, excitons and electrons can be obtained using JIP-test analysis from OJIP curves (Appenroth et al. 2001). A simplified model of the electron transport in PSII (Strasser et al. 1995) is proposed (Fig. 2). In the model, ABS refers to photon flux absorbed by the antenna pigments and excited chlorophyll, Chl*. Part of the excitation energy is dissipated mainly as heat and less as fluorescence emission F, and another part is trapped as trapping flux TR to the RC to be converted into redox energy by reducing the electron acceptor Q_A to Q_A^-, which is then re-oxidized to Q_A by donating an electron to Q_B. This is how an electron transport (ET) is created which ultimately leads to CO_2 fixation.

For the dark-adapted sample, all RCs were assumed to be open when all Q_A were in their oxidized forms and could

Table 1 Formulae and terms used in the JIP-test (Strasser et al. 2000)

Formulae and terms	Illustrations
$V_J = (F_{2ms} - F_o)/(F_m - F_o)$	Relative variable fluorescence intensity at the J-step
$M_o = 4(F_{300\mu s} - F_o)/(F_m - F_o)$	Approximated initial slope of the fluorescence transient
$S_m = (\text{Area})/(F_m - F_o)$	Multiple turn-over in the closure of the reaction centers.
$S_S = V_J/M_o$	The smallest S_m, single turn-over
$N \equiv S_m/S_S = S_m \cdot M_o \cdot (1/V_J)$	Turn-over number Q_A
Quantum efficiencies or flux ratios	
$\varphi_{Po} = TR_o/ABS = [1 - (F_o/F_m)] = F_V/F_m$	Maximum quantum yield for primary photochemistry
$\varphi_{Eo} = ET_o/ABS = [1 - (F_o/F_m)] \cdot \psi_o$	Quantum yield for electron transport (at t = 0)
$\varphi_{Do} = 1 - \varphi_{Po} - (F_o/F_m)$	Quantum yield for dissipated energy
$\psi_o = ET_o/TR_o = 1 - V_J$	Probability that a trapped exciton moves an electron into the electron transport chain beyond QA (at t = 0)
Specific fluxes or specific activities	
$ABS/RC = M_o \cdot (1/V_J) \cdot (1/\varphi_{Po})$	Absorption flux per reaction center
$TR_o/RC = M_o \cdot (1/V_J)$	Trapped energy flux per reaction center (at t = 0)
$ET_o/RC = M_o \cdot (1/V_J) \cdot \psi_o$	Electron transport flux per reaction center (at t = 0)
$DI_o/RC = (ABS/RC) - (TR_o/RC)$	Dissipated energy flux per reaction center (at t = 0)
Performance indexes	
$PI_{ABS} = (RC/ABS) \cdot [\varphi_{Po}/(1 - \varphi_{Po})] \cdot [\psi_o/(1 - \psi_o)]$	Performance index on absorption basis

ABS the energy fluxes for absorption, *TR* the energy fluxes for trapping, *ET* the energy flux for the electron transfer from QA to QA^-

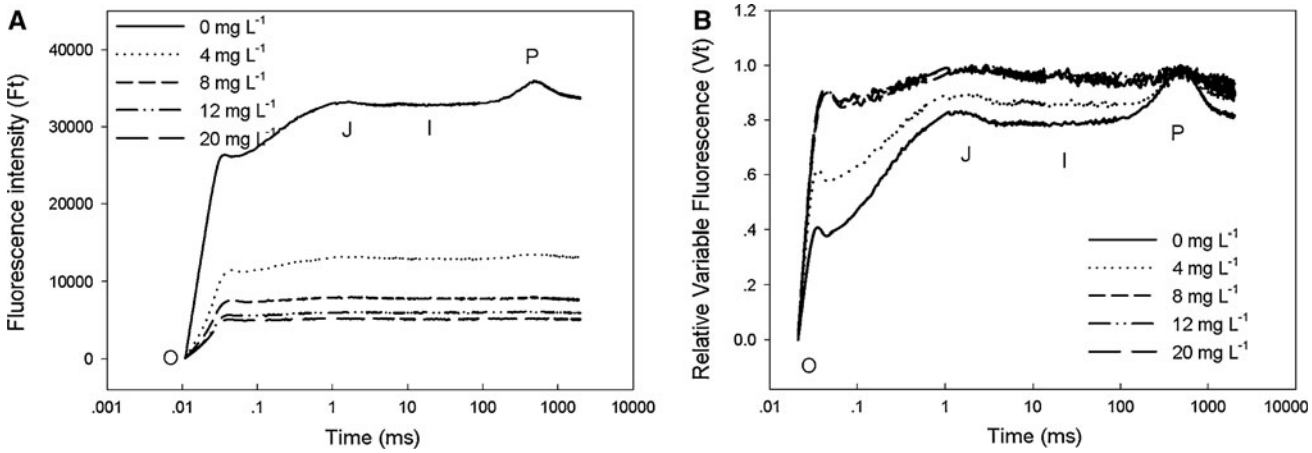

Fig. 1 The fast chlorophyll fluorescence curves of *M. aeruginosa* after 72 h treatment with artemisinin at different concentrations. F_t represents fluorescence intensity at time t; V_t represents the relative variable fluorescence at time t, $V_t = (F_t - F_o)/(F_m - F_o)$

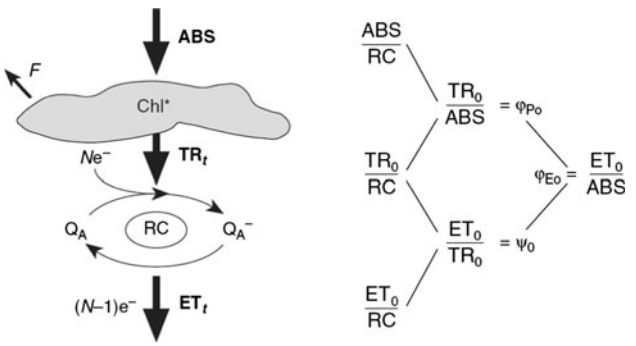

Fig. 2 A highly simplified working model of the energy fluxes of PSII (Strasser et al. 1995)

promote the reduction of Q_A to Q_A^-. If Q_A is reduced, then the RC is called closed. V_J is a measure of the fraction of primary quinine electron acceptor Q_A at its reduced state and also represents a fraction of closed RCs (Strasser et al. 1995). Conversely, ψ_o ($\psi_o = 1 - V_J$) reflects the opening of RCs. F_v/F_m is called maximum quantum yield of primary photochemistry, which reflects the ratio of variable fluorescence to maximal fluorescence. In our study, Fig. 3 shows that V_J and F_v/F_m increased gradually with artemisinine concentration, V_J increasing from 0.8 to about 1.0, and F_v/F_m rising from around 0.4 to 0.6. ψ_o on the other hand decreased with increasing artemisinin concentration. The increased V_J and decreased ψ_o indicate that Q_A^- accumulation at J and I points

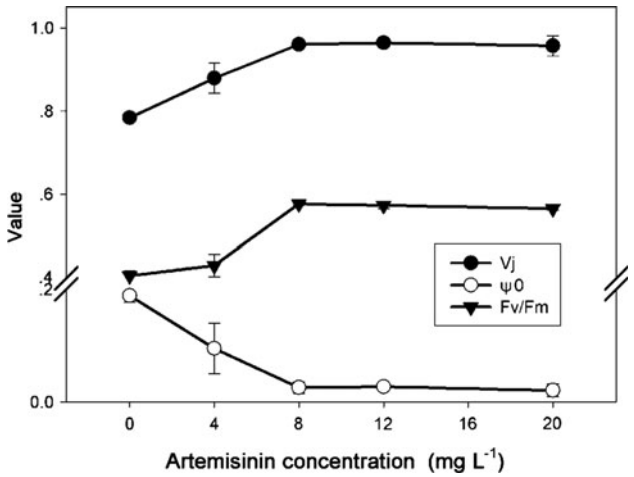

Fig. 3 Effect of artemisinine on parameters (V_J, ψ_o, F_v/F_m) from JIP-test of *M. aeruginosa* after 72 h treatment with artemisinin at different concentrations. Data are mean ± SD (n = 4)

(after illumination at 2 ms and 30 ms) was a result of artemisinine inhibition of electron transfer from Q_A^- to Q_B. Furthermore, the decrease in the efficiency of electron transfer from Q_A^- to Q_B (ψ_o) at high artemisinine concentration indicated an inhibition of Q_A^-. The results showed that the acceptor side in the electron transport of PSII was a target site of artemisinine stress.

Artemisinin stress had little effects on the minimum value of Sm (Ss) but had greater impact on the rate of oxidation and reduction of Q_A (Mo), Sm and N. Mo increased with artemisinin concentration, but Sm and N tended to decrease (Fig. 4). It was apparent that the parameters (Sm, Ss, N and Mo) bind to each other. The parameter Sm is a measure of the energy needed to close all RCs. Subscript "m" refers to the multiple turnover for the RCs" closure. The more the electrons from Q_A^- are transferred into the electron transport chain ET, the longer the fluorescence signals remain lower than F_m and the bigger Sm becomes (Srivastava et al. 1998; Strasser et al. 2000). The smallest possible normalized total area corresponds to the case when each Q_A is reduced only once, as in the presence of DCMU, and it can then be denoted as Ss indicating single turnover. Therefore, the turn-over number N (N = Sm/Ss), indicated how many times Q_A had been reduced in the time span from 0 to t_{Fmax} (Srivastava et al. 1998). The value of Sm and N could reflect the size of PQ pools. If an exponential fluorescene rise for the single turn-over situation, then the normalized area Ss would be inversely proportional to the initial slope of the relative variable fluorescence. However, the value of this slope can be calculated utilizing only data from the in vivo fluorescence transient as Mo without the requirement of additional measurements in the presence of DCMU (Strasser et al. 1995; Srivastava et al. 1998; Strasser et al. 2000). The results in this study indicated that Q_A reduction rate increased, but the energy of reducing a single Q_A remained unchanged; On the other hand, since Sm decreased, i.e. PQ storage capacity of PS II reaction Center receptor side decreased, the corresponding redox number of QA also reduced.

Figure 5 shows that with increasing concentration of artemisinin φ_{Po} also had an increasing trend whereas TRo/RC remained virtually unchanged. On the other hand; ψ_o, φ_{Eo}, ABS/RC, ETo/RC all had decreasing trends. In JIP test, the fluxes of φ_{Po}, ψ_o, φ_{Eo} and φ_{Do} were directly related with each other, which were called quantum efficiencies or flux ratios. The maximum quantum yield of primary photochemistry φ_{Po} ($\varphi_{Po} \equiv$ TRo/ABS = Fv/Fm), the efficiency that a trapped exciton can move an electron further than Q_A^- into the electron transport chain ψ_o ($\psi_o \equiv ET_o/TR_o$), or the probability that an absorbed photon will move an electron into the electron transport chain ψ_o ($\psi_o \equiv ET_o/TR_o$), are directly related to the three fluxes, as the ratios of any two of them (Strasser et al. 2000). The specific energy fluxes ABS/RC,

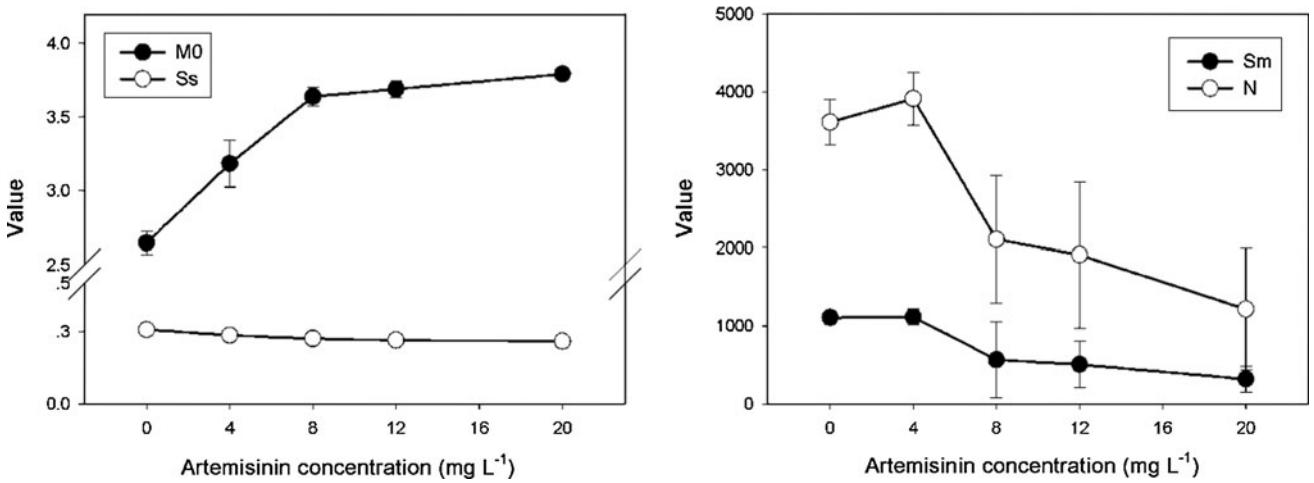

Fig. 4 Effect of artemisinine on parameters (Ss, Mo, Sm, N) from JIP − test of *M. aeruginosa* after 72 h treatment with artemisinin at different concentrations. Data are mean ± SD (n = 4)

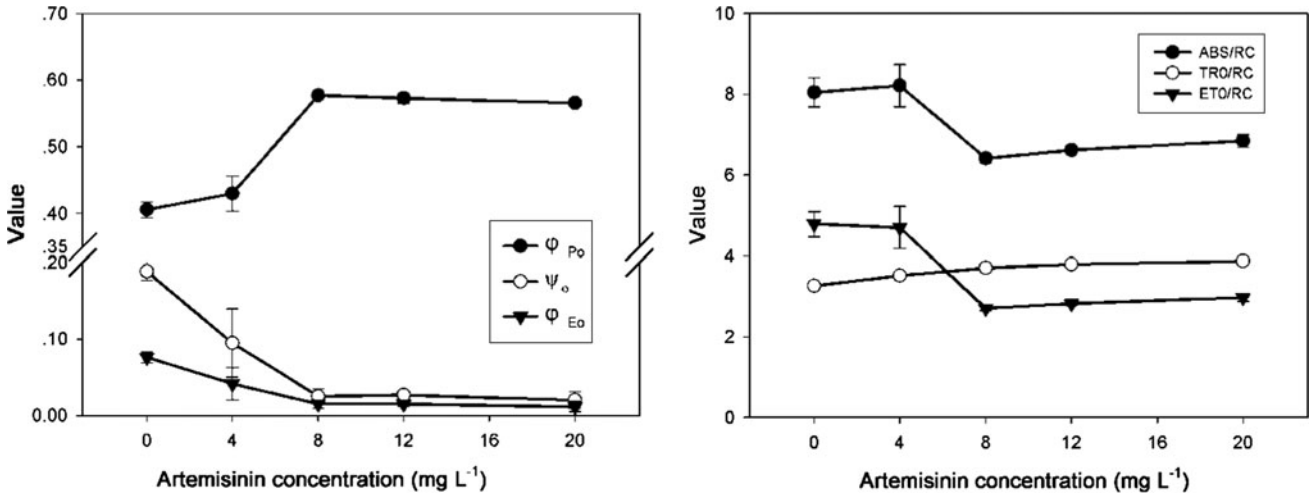

Fig. 5 Effect of artemisinine on quantum efficiencies and specific activities of *M. aeruginosa* after 72 h treatment with artemisinin at different concentrations. Data are mean ± SD (n = 4)

ETo/RC and TRo/RC can be derived from the parameters of φ_{Po}, ψ_o, φ_{Eo}. Since only red actinic light has been used, the absorption is proportional to the chlorophyll concentration. The expression ABS/RC can be taken as a calculated average amount of chlorophyll which channels excitation energy into RC. Therefore, ABS/RC can be taken as a measure for an average antenna size. TR/RC expresses the efficiency of trapped excitons by the RC resulting in the reduction of Q_A to Q_A^-. The maximal value of this efficiency is given by TRo/RC, because at time zero all RCs are open. It has to be pointed out that TRo/RC expresses the initial rate of the closure of RCs as a fractional expression over the total number of RCs that can be closed. It is possible that under artemisinin stress some RCs are inactivated in the sense of being transformed to quenching sinks (Krause et al. 1990) without reducing Q_A to Q_A^-. Therefore our study showed, although the trapped energy from absorption of Chl* (φ_{Po}) was rising, the efficiency of trapped excitons (TRo/RC) was unchanged. The decreased value of ψ_o, φ_{Eo}, and ETo/RC reflect an inhibition of the downstream of Q_A^-. Therefore the electron transport from Q_A^- to Q_B had been inhibited under the artemisinin stress.

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