



The effect of orange g dyeing wastewater on photosynthesis and antioxidant enzyme activities of *Acorus calamus*

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Abstract

In order to explore the tolerance of *Acorus calamus* on Orange G dyeing wastewater and the feasibility of its remediation of dyeing wastewater pollution, we determined photosynthesis, chlorophyll fluorescence parameters and antioxidant enzymes activity of *Acorus calamus* in nine dye concentrations (0, 60, 120, 180, 240, 300, 360, 420 and 480 mg·L⁻¹) using hydroponics. Orange G inhibited the photosynthesis of *Acorus calamus*, which presented as decreased net photosynthesis rate (P_n), maximum quantum yield of PSII (F_v/F_m) and actual photochemical efficiency (Φ_{PSII}). P_n and stomatal conduction (G_s) decreased and intercellular CO₂ concentration (C_i) increased with increasing concentration of Orange G, showing that the main reason for restricting P_n had turned into non-stomatal limitation; The activities of the antioxidant enzyme were activated in the dye concentration below 120 mg·L⁻¹, and the antioxidant enzyme system was damaged in the dye concentration above 120 mg·L⁻¹. These results implied that function of photosynthetic apparatus in *Acorus calamus* seedling leaves was maintained by a synergistic effect of thermal energy dissipation and regulating the antioxidant enzymatic activity jointly. Non-stomatal limitation responsible for reduction in P_n was associated with the impairment of PS II and antioxidant enzyme system when dye concentration was above 120 mg·L⁻¹.

Keywords: orange g, photosynthetic physiology, *Acorus calamus*, antioxidant enzyme activity

1. Introduction

Since the first commercial dye was artificially synthesized (Rai *et al.*, 2005) [23], there are tens of thousands synthetic dyes have been found and used (Robinson *et al.*, 2001) [26]. Although synthetic dyes bring huge economic benefits, they caused many environmental problems, mainly on the water environment pollution. The most widely used synthetic dyes are azo dyes, because of their low production cost, the advantages of high stability, a variety of color and used in textile, leather, paper, food, cosmetics and pharmaceutical industry (Pandey *et al.*, 2007; Jin *et al.*, 2007; Chang *et al.*, 2004; Telke *et al.*, 2008) [9, 12, 28, 29]. Under natural environment, azo dyes can be broken down to produce more than 20 carcinogenic aromatic amines which alter the DNA of the human body structure, cause lesions and cause cancer. Orange G (OG) is a typical azo dyestuff, discharging waste water containing azo dyes and their metabolites in aquatic ecosystems can make the water quality deterioration, lower the opacity of the water and dissolved oxygen content, increase water stunk, water color, etc., at the same time also has toxic effects to the aquatic animals and plants, inhibit the growth of its breeding, lead to a more serious problem of water environment (Vandevivere *et al.*, 1998) [30], especially the water security problem.

A wide variety of dye waste water purification methods are mainly concentrated in the physical and chemical methods, but due to large projects of those methods, those method sare high cost. Bioremediation method has been proven to remove the dyestuff, but its processing product maybe cause secondary pollution to the environment (Khandare *et al.*, 2013) [14]. Phytoremediation is mainly using plant physical characteristics to absorption and degradation of dye, to

decompose pollutants into non-toxic harmless substance, and the cost is relatively low (Dawkar *et al.*, 2008; Cluis, 2004) [6, 5], so it's widely used recently (Kabra *et al.*, 2011) [13]. Mahmood found that cattail could make reactive blue 19dye decolorization rate between 50% and 75%, which was better than that of anaerobic sludge (Mahmood *et al.*, 2014) [16].

Acorus calamus is a perennial herbaceous plant, the plant distributed in all parts of China (Ren Qifei *et al.*, 2013). Zhang Weihao studied *Acorus calamus* on verdigris microcystin algae (*Microcystis aeruginosa*) of allelopathic inhibition, their results show that the calamus can inhibit the growth of microcystin algae, etc. (Zhang weihao *et al.*, 2006) [31]. Zhang (Zhang *et al.*, 2016) [34] found that orris root extract on the growth of cyanobacteria have good inhibitory effect. Calamus has better fouling resistance and barren resistance, it has a good application prospect in water ecological restoration engineering (Wu xiaogang, 2006). There fore calamus was selected as hydroponics experimental material to measure the ratios of different concentration of OG wastewater on calamus leaf photosynthetic physiological effects and the activity of antioxidant enzymes in leaves and roots. On the basis of understanding the tolerance of calamus to different concentration of OG, it maybe be used to purify printing and dyeing wastewater.

2. Materials and Methods

2.1 Materials and experimental design

The experiment was conducted in the greenhouse at Shandong Agricultural University. Potted calamus seedlings was purchased from a flower production company. They

were placed in the green house for two weeks to adapt to the growing environment. Then, 36 calamus bare root seedlings were transplanted in 250 mL conical beakers containing half strength Hoagland's hydroponic nutrient solution. Those conical beakers covered by black plastic film to deter algal growth. The seedlings were cultured two weeks in the greenhouse.

Then, the decolorization of wastewater containing Orange G by calamus seedlings was conducted. One seedling was placed in a conical beaker containing 250 mL half strength Hoagland's solution only (control treatment) or spiked 60, 120, 180, 240, 300, 360, 420 and 480 mg·L⁻¹ Orange G. Each treatment has four replicates. Mark at the top of the solution on the bottle, every other day half strength hoagland and nutrient solution was filled into the bottle to the marked line. In the 7th and 13th day after stress treatment, determination of photosynthetic physiological parameters and chlorophyll fluorescence parameters of plant leaves was conducted. Finally, leaves were collected and stored in liquid nitrogen for the determination of enzyme activity.

2.2 Index and its measuring method

2.2.1 Photosynthetic physiological parameters determination

In sunny day, net photosynthetic rate (P_n), transpiration rate (T_r), stomatal conductance (G_s), intercellular CO₂ concentration (C_i) and air CO₂ concentration (C_a) was determined with CIRAS-2 photosynthesis system (PP Systems, UK). During the measurements, the photosynthetic active radiation (PAR) was set 1200 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ by artificial light (light emitting diode, LED). Water use efficiency (WUE) and stomatal limitation (L_s) was calculated by the equations: $WUE=P_n/T_r$ (Nijs *et al.*, 1997) [18], the $L_s=1-C_i/C_a$ (Berry J A, *et al.*, 1982) [1].

2.2.2 Chlorophyll fluorescence parameters determination

Chlorophyll fluorescence parameters were measured using pulse modulation fluorescence system (FMS2.02 type, British Hansatech). Minimal fluorescence yield of the light-adapted state (F_o), steady-state fluorescence yield (F_s) and maximal fluorescence yield of the light-adapted state (F_m) were measured between 8:30 -11:30 in the morning. Minimal fluorescence (F_o) and the maximal fluorescence (F_m) were determined after 30 min dark adaptation. Some other Chl fluorescence parameters were calculated according to the formula as following:

maximum photochemical efficiency of PS II: $F_v/F_m=(F_m-F_o)/F_m$; PS II actual photochemical efficiency: $\Phi_{PSII}=(F_m-F_o)/F_m$; Photochemical quenching coefficient: $qP=(F_m-F_s)/(F_m-F_o)$; Non photochemical quenching: $NPQ=(F_m-F_m')/F_m'$ (Krause *et al.*, 1991; Rohacek, 2002) [15, 27].

2.2.3 The determination of antioxidant enzyme activity

Superoxide dismutase (SOD) activity assayed by measuring its ability to inhibit the photochemical reduction of nitro blue tetra-zolium (NBT) following the method of Giannopolitis and Ries (Giannopolitis *et al.*, 1977) [8]. One unit of SOD activity was defined as the amount of enzyme that caused 50% inhibition of photochemical reduction of NBT. SOD activity was expressed as U mg⁻¹protein. Peroxidase (POD) activity was performed by guaiacol chromogenic method according to Cakmak and Marschner (Cakmak I *et al.*, 1992) [2].

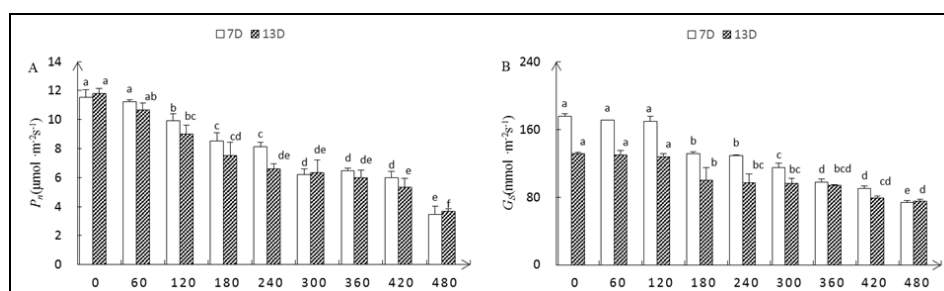
2.3 Data analysis

Excel 2013 software and SPSS19.0 were used for data analysis and mapping. The difference between control and treatments were analyzed by one-way analysis of variance (ANOVA) and Duncan's multiple-range test was used for multiple comparisons.

3. Results

3.1 Effects of OG on photosynthetic physiological parameters of calamus

In the seventh day and thirteenth day after OG exposure, with the increase of orange G concentration, P_n , G_s , L_s and WUE showed a downward trend, however, C_i showed an upward trend (Fig. 1). The T_r increased at lower OG concentration (≤ 120 mg·L⁻¹) and decreased at higher OG Concentration (≥ 180 mg·L⁻¹). In seventh day, when the concentration of OG was equal or greater than 120 mg·L⁻¹, there was significant difference of P_n between the treatment group and the control ($P<0.05$). For G_s , C_i , T_r , L_s and WUE, the critical concentration of OG was 180, 480, 60, 180 and 60mg·L⁻¹ respectively. In the thirteenth day, when the concentration of OG was equal or greater than 120 mg·L⁻¹, there was significant difference of P_n between the treatment group and the control ($P<0.05$). For G_s , T_r , L_s and WUE, the critical concentration of OG was 180, 60, 60 and 60mg·L⁻¹ respectively. When dye concentration reached 420 mg·L⁻¹, P_n dropped to 51.99% to the control group in seventh day. In the seventh day and thirteenth day, the P_n and G_s showed downward trend, on the contrast, C_i showed an upward trend. Hence, based on Farquhar and Sharkey (Farquhar, *et al.*, 1982) Criterion of discrimination of photosynthesis limitations, the main reason that led to the decrease of the P_n is from non stomatal factors.



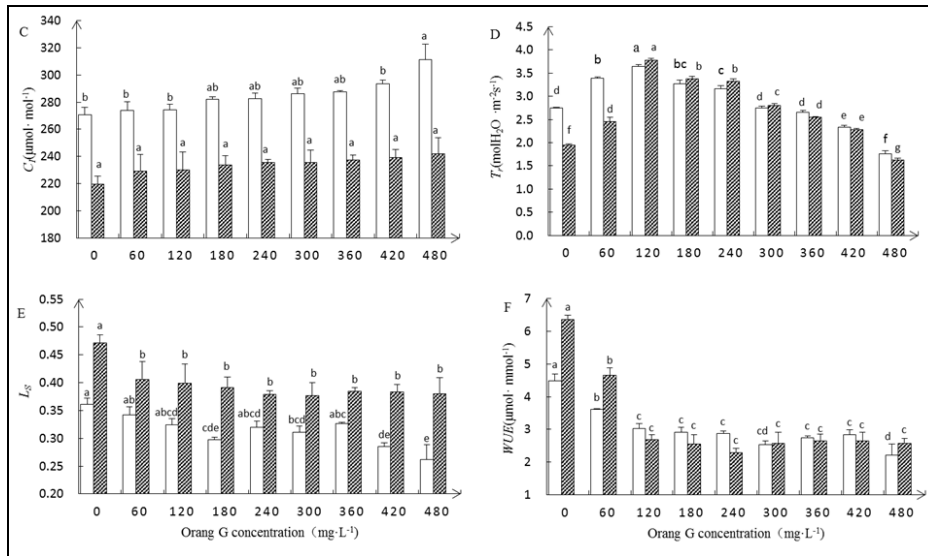


Fig 1: The response of photosynthetic physiological parameters of *Acorus calamus* to Orange G wastewater.

Different lower case letters indicate that means are significantly different from each other ($p < 0.05$).

2.2 Effects of OG dye stress on chlorophyll fluorescence parameters of calamus

Fig. 2 shows that under the stress of different concentration of dye, the minimal fluorescence (F_o), the effective quantum yield of PSII photochemistry (Φ_{PSII}), maximum fluorescence (F_m) and Photochemical quenching coefficient (qP), the maximal quantum yield of PSII photochemistry (F_v/F_m) and Non Photochemical quenching (NPQ) in leaves

of the calamus have obvious change. In the seventh day and thirteenth day after OG exposure, with the increase of orange G dye concentration, Φ_{PSII} , F_m , qP , F_v/F_m and NPQ showed a downward trend, but F_o showed an upward trend. When the concentration of OG was greater than $240 \text{ mg}\cdot\text{L}^{-1}$, there was significant difference of F_o and qP between the treatment group and the control ($P < 0.05$). For Φ_{PSII} , F_m , F_v/F_m and NPQ , the critical concentration of OG was 420, 180, 360, 300 $\text{mg}\cdot\text{L}^{-1}$ respectively. There was no significant difference between the same parameter in seventh day and thirteenth day.

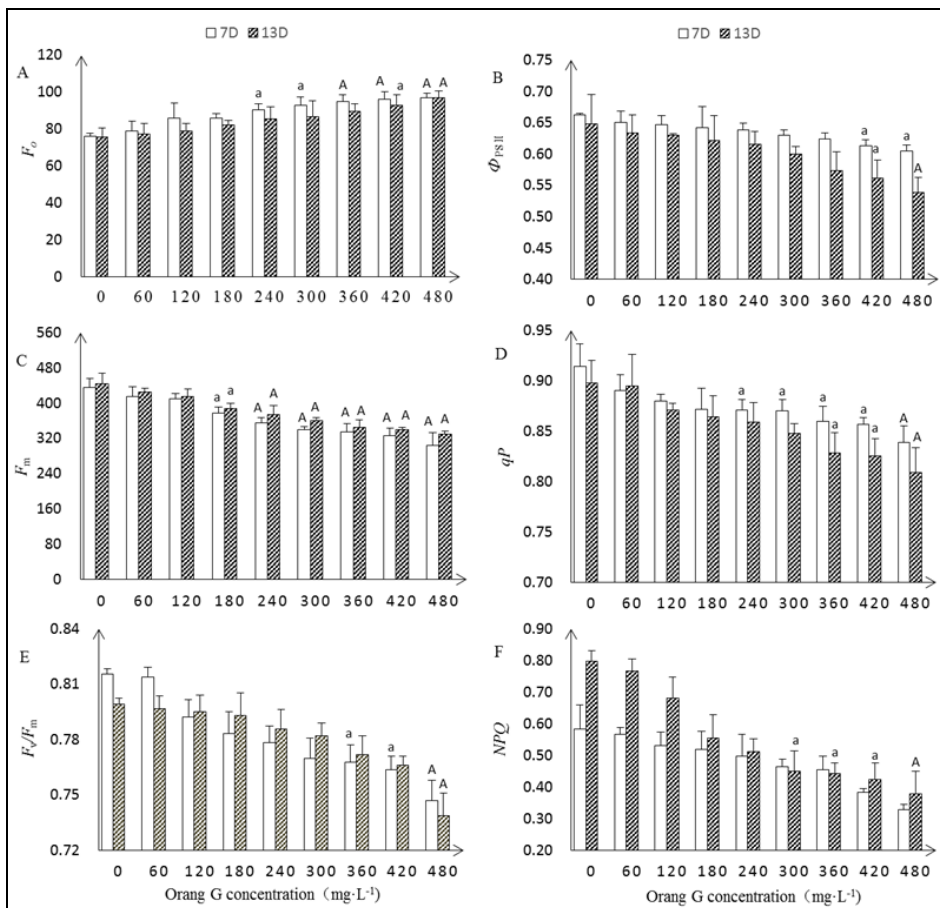


Fig 2: Fluorescence parameters of *Acorus calamus* under the orange G dye stress.

Letter a means that the differences were significant at 0.05 level. Letter A means that the differences were significant at 0.01 level. 2.3 Effect of OG dye stress on the activity of antioxidant enzymes with the increase of dye concentration, the POD and SOD activity in roots and leaves increased at lower dye concentration ($\leq 120 \text{ mg}\cdot\text{L}^{-1}$) and decreased at higher dye concentration ($\geq 180 \text{ mg}\cdot\text{L}^{-1}$) (Fig. 3). The difference of SOD activity in root between the control and the treatment was significant when the dye concentration

was $420 \text{ mg}\cdot\text{L}^{-1}$ ($P < 0.01$). The difference of SOD in leaves and POD in leaves and roots was significant when the dye concentration was equal or greater than $360 \text{ mg}\cdot\text{L}^{-1}$ ($P < 0.05$). When the concentration of OG reached $480 \text{ mg}\cdot\text{L}^{-1}$, POD and SOD activity in roots and leaves reached the minimum value, POD activity in roots and leaves only 37.86% and 37.86% of control respectively, SOD activity in roots and leaves only 42.93%, 44.06% of control respectively.

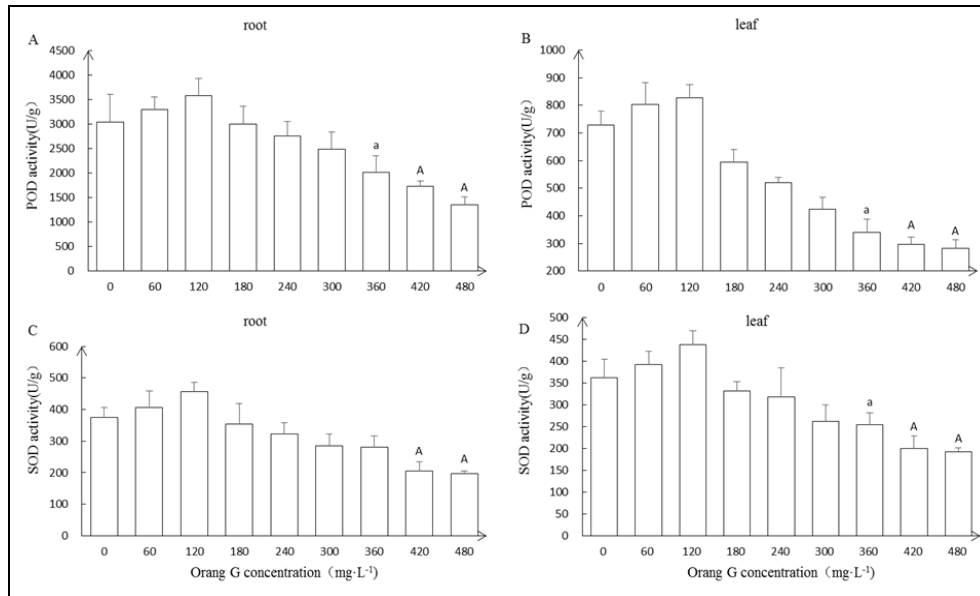


Fig 3: Under the orange G dye stress, antioxidant enzyme activity changes in root and leaf of *Acorus calamus*

Values represent Mean \pm S.E.M = Mean values \pm Standard error of means, n=3. Letter a means that the differences were significant at 0.05 level. Letter A means that the differences were significant at 0.01 level.

4. Discussion

Phytoremediation is an effective method using plants to remove and reduce or fixed pollutants in soil and water, no secondary pollution to the environment in the governance process (Pilon - Smits, 2005) [21]. Chang *et al.* found that in $\text{NO}_3^- / \text{NH}_4^+$ ratio between 50/50 and 75/25 calamus is more suitable for the construction of wastewater treatment wetland (Chang J *et al.*, 2010) [4]. Ren Jun *et al.* reported that calamus has the strongest absorption capacity of Cd and the reeds has the weakest absorption capacity of Cd (Ren jun *et al.*, 2010). The results in this study show that (Fig. 1), OG dye has inhibitory effect to the photosynthesis of calamus and with the increase of dye concentration, the inhibition enhances. Many researches reported when stress factor aggravated, the main reason for the photosynthesis reduction was transition from stomatal limitation to non-stomatal factors (Farquhar, *et al.*, 1982; Zhang *et al.*, 2010; Pei bin, *et al.*, 2013). The results in this study found that in the seventh day and thirteenth day, when the concentration of OG was equal or greater than $120 \text{ mg}\cdot\text{L}^{-1}$, there was significant difference of Pn between the treatment group and the control ($P < 0.05$). Hence, it can be inferred that $120 \text{ mg}\cdot\text{L}^{-1}$ is an approximate critical concentration that causes the main reason of reduction of photosynthesis of calamus changed from stomatal factors to non-stomatal factors. In the concentration of $60\text{-}120\text{mg}\cdot\text{L}^{-1}$, the accurate critical concentration still needs to be further explored.

Photochemical quenching (qP) reflects the pigment absorption of light energy is used for PS II antenna photochemical electron transfer, to some extent, reflects the PS II reaction center's openness. The greater its value, the higher the electron transfer activity of PS II (Guo *et al.*, 2009; Han gang *et al.*, 2010; Gilmore *et al.*, 1991) [10, 9]. Non photochemical quenching (NPQ) is PS II antenna pigment absorption of wasted energy in the form of heat is a protective mechanism of photosynthetic mechanism, characterization of PS II reaction center of antenna after the pigment absorbs excess light energy, heat dissipation capacity and the damage degree of photosynthetic mechanism (Gilmore *et al.*, 1991) [9]. The F_v/F_m variable can be a good indicator of the performance of the photosynthetic apparatus and shows the ability of the plants to tolerate environmental stresses (Maxwell and Johnson 2000) [17]. In this study, F_m and F_v/F_m decreased, suggests that photoinhibition occurred in calamus and at the same time F_o rising shows that PS II was damaged (Xu *et al.*, 1999; Xu *et al.*, 2005) [32, 33]. Φ_{PSII} , F_m , qP , F_v/F_m and NPQ reduced in different degree and qP decrease obviously, qP reduction shows the reduction activity of PS II reaction center (Pinol R *et al.*, 2009; Zhu yinghua, *et al.*, 2011) [22, 38]. F_v/F_m and Φ_{PSII} declining means that in the dark or light conditions and the actual photochemical efficiency is lower. Therefore, dye treatment can significantly inhibit or reduce the PS II photochemical efficiency and activity in leaves of calamus. Changes of activity of antioxidant enzyme system and membrane lipid peroxidation in plant have been widely used in the researches on the mechanism of the plant to respond to adversity. Under adverse environment, the process of

vegetation carbon assimilation and the activity of PS II reduced, leading to excitation energy rise and cause the excess energy in generation of reactive oxygen species and the clearance balance is broken, a substantial accumulation of free radicals in the body, the cause of membrane lipid peroxidation, cause product membrane lipid peroxide (MDA) increased, and reduces the photosynthetic capacity of the plant leaves (Sohrabi *et al.*, 2012) [28]. Plants under stress induced energy to start their own protection mechanism, to minimize damage. SOD is a naturally occurring, O₂⁻ scavenging convert's harmful O₂⁻ group into H₂O₂ and O₂ by catalyzing the O₂⁻. CAT and POD can immediately catalyze it into completely harmless water (Zhou *et al.*, 2013) [25]. In plants under adversity stress, the phenomenon of SOD and POD activity increased is a kind of self protection mechanism of plant resistance to stress. This study found that POD and SOD activity in roots and leaves increased at lower dye concentration ($\leq 120 \text{ mg}\cdot\text{L}^{-1}$) and decreased at higher dye concentration ($\geq 180 \text{ mg}\cdot\text{L}^{-1}$), which indicated that low concentration of dye could obviously promote the activity of SOD and POD in calamus, while the high concentration dye will inhibit the activity of SOD and POD in calamus.

5. References

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