Phytotoxicity of atrazine combined with cadmium on photosynthetic apparatus of the emergent plant species *Iris pseudacorus*

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Abstract

The combined pollution, instead of single pollution, has become a widespread contamination phenomenon in aquatic environment. However, little information is now available about the joint effects of the combined pollution, especially co-existed pesticides and heavy metals, on aquatic plants. In the present study, using continuous excitation chlorophyll fluorescence parameters and the OJIP transient, comparisons of herbicide atrazine (ATZ) phytotoxicity on *Iris pseudacorus* between in the presence and absence of cadmium (Cd) were evaluated over an exposure period of three weeks under laboratory conditions. Results showed that both ATZ and Cd were toxic to *I. pseudacorus*. The ratio F_v/F_o , specific electron transport energy (ET_0/RC) and photochemistry efficiency (PI_{abs} and PI_{total}) of this emergent plant species at individual ATZ and Cd concentrations were significantly lower than those of the control. ATZ mainly inhibited electron transport beyond Q_A at PSII acceptor side as indicated by the sharp rise of the J-step level of fluorescence rise kinetics. A pronounced K-step and the loss of I-step due to the damage on the OEC and PSI also occurred when ATZ was at or above $1.0 \text{ mg} \cdot \text{L}^{-1}$. In comparison to ATZ alone, ATZ combined with Cd resulted in a lower amplitude rise in J-step with apparent J-I and I-P phases; and significantly lower F_o with higher F_v/F_o , as well as greater ET_0/RC with higher values of PI_{abs} and PI_{total} . However, the adverse influences of ATZ combined with Cd on the above indicators were still significant as compared with the control. Therefore, the coexistence of Cd alleviated the individual phytotoxicities of ATZ, whereas combined pollution of ATZ and Cd still induced the decline in photosynthetic performance of *I. pseudacorus*, and its potential ecological impacts on the aquatic vegetation cannot be ignored. Our findings offer a better understanding of the joint effects of the pesticide and heavy metal on non-target aquatic plants, and provided valuable insights into the interaction of these pollutants in aquatic environment.

Introduction

Atrazine (ATZ), a triazinic herbicide, is the second most widely consumed pesticide worldwide (Singh et al. 2018). Due to its extensive and long-term application, ATZ has become a widespread pollutant in water environments with an expected environmental concentration of 2.7 mg L^{-1} (Peterson et al. 1994; Albright et al. 2013). The lowest observed effect concentration of ATZ for the growth of emergent plant is 2 mg L^{-1} (Wang et al. 2014). Exposure to various herbicides in surface waters is one of the main factors responsible for the loss of native plant vegetation (Knauert et al. 2010). Additionally, ATZ is still a subject of continuous concern due to its potential endocrine and carcinogenic activity (Jablonowski et al. 2011). Meanwhile, Cd is a kind of typical heavy metal pollutant in aquatic systems (Xin et al. 2020). The amount of Cd occurring in water is generally less than 1 mg L^{-1} (Das and Jana 2004; Bhardwaj et al. 2020), whereas extraordinarily high concentrations of total Cd up to 5 mg L^{-1} in waters have been reported (Thornton and Walsh 2001). Such concentrations potentially pose a threat to human health because of its harmful effects on the food chain (Das and Jana 2004). Furthermore, considering that the co-presence of pesticides and heavy metals in aquatic systems has become a widespread contamination phenomenon, interactions between them may influence the fate of these pollutants, thus the

ecotoxicological effects of one contaminant may be enhanced (synergism) or eliminated (antagonism) due to the presence of others (Starling et al. 2019).

To date, only a small number of studies reported the joint biological toxicity of ATZ and Cd. These studies indicated that ATZ combined with Cd showed less than additive toxicity to the earthworm (*Eisenia fetida*) (Wang et al. 2012; Yu et al. 2019) and rice (*Oryza sativa*) (Su et al. 2005) owing to the formation of ATZ/Cd complexes. Yet, none of these investigations addressed the joint toxicity of these contaminant mixtures to aquatic plants which are of great importance in stabilizing function and structure of freshwater ecosystems. In addition, ATZ in coexistence with Cd displayed a prolonged half-life in water (Xie et al. 2021). The prolonged persistence of ATZ implies an increase in potential ecological risks. Therefore, there is a clear need to focused on the combined effects of ATZ and Cd on aquatic plants. It is well established that ATZ is an inhibitor of photosynthesis (Gronwald et al. 1989). Also, Cd inhibits electron transfer reactions in PSII (Sigfridsson et al. 2004). Moreover, photosynthesis is the basic physico-chemical process for plant growth and survival, and is usually considered as a top priority in probing toxicological action of a compound toward plants (Gao et al. 2018). Thus, photosynthetic characteristics of plants can provide more direct and fundamental evidences for joint action induced by these two photosynthesis inhibitors. *Iris pseudacorus*, a completely emergent plant with high ornamental value, is commonly distributed in nature and artificial water bodies, and has been frequently used for phytoremediation of wastewaters. The purpose of this study was to evaluate the combined effects of ATZ and Cd on photosynthesis performance of *I. pseudacorus* through chlorophyll *a* fluorescence induction OJIP transients. To accomplish the objective, the present study investigated the changes of chlorophyll fluorescence parameters and differential kinetics in this plant species under ATZ stress in the presence and absence of Cd over an exposure period of three weeks.

Materials And Methods

Plant materials and chemicals

Seeds of *Iris pseudacorus* were sterilized with sodium hypochlorite solution for 10 min, followed by thorough washing with deionized water. The seeds were germinated in moist peat soil for five weeks, uniform seedlings were selected and transferred to flasks (one plant per flask) containing 250 ml 10% Hoagland nutrient solution. The seedlings were acclimatized for one week with the culture solution prior to the start of the experiments.

ATZ, used as a formulated product (90% water dispersible granules, BUGAO®), was purchased from Zhongshan Chemical Industry Co., Ltd., China. Cd was used as Cd(NO₃)₂·4H₂O which was of analytical grade with 99% purity.

Experiment treatment

According to concentrations in the previous studies and the special case ever reported, the ranges in aqueous exposure concentrations of ATZ were 0.1, 0.5, 1.0, and 2.0 mg·L⁻¹, concentrations of Cd were 5.0

and 10.0 mg L^{-1} , which fall within the reported field and research concentrations. The experimental design was as follows:

- (1) treatment with ATZ alone: 10% Hoagland nutrient solution with ATZ at concentrations of 0.1, 0.5, 1.0, and $2.0 \text{ mg}\cdot\text{L}^{-1}$ without added Cd;
- (2) treatment with Cd alone: 10% Hoagland nutrient solution with Cd^{2+} (as $\text{Cd}(\text{NO}_3)_2\cdot 4\text{H}_2\text{O}$) concentrations of $5.0 \text{ mg}\cdot\text{L}^{-1}$ and $10.0 \text{ mg}\cdot\text{L}^{-1}$ without added ATZ;
- (3) Combined treatment: 10% Hoagland nutrient solution with ATZ at concentrations of 0.1, 0.5, 1.0, and $2.0 \text{ mg}\cdot\text{L}^{-1}$, and with added Cd^{2+} (as $\text{Cd}(\text{NO}_3)_2\cdot 4\text{H}_2\text{O}$) at concentrations of 5.0 and $10.0 \text{ mg}\cdot\text{L}^{-1}$, respectively;
- (4) Control: 10% Hoagland nutrient solution without both ATZ and Cd.

Each treatment had three replications, and consisted of one plant per replicate in a flask holding 250 mL treatment solution. The experiments were carried out in a controlled environment growth chamber with a 14-h light period ($350 \mu\text{mol m}^{-2} \text{ s}^{-1}$) and a temperature cycle of 25/20 °C day/night. The relative humidity was 70%. The culture nutrient solution was supplemented every 24 h to a volume of 250 mL (initial solution volume) to compensate for the nutrition consumption and water vaporization, and was replaced once a week in order to maintain a relatively constant ATZ level across the entire experiment. The chlorophyll fluorescence parameters were measured after three weeks of continuous cultivation.

Chlorophyll fluorescence measurement

Chlorophyll fluorescence measurements were performed in each plant with a pocket fluorometer (Handy PEA, Hansatech, UK). The light intensity was $3000 \mu\text{mol (photons)}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$, and the leaves were dark-adapted for 30 min before measurements were started. For each treatment, 9 measurements (three replicates, three leaves in each replicate) were made. The chlorophyll fluorescence emission induced between 10 μs and 2 s by the light pulses was digitized by the instrument. The selected JIP-test parameters used to characterize the photosynthetic performance were listed in Table 1 (Guo et al. 2020; Sun et al. 2020; Liu et al. 2020). These parameters derived from the original fast fluorescence transient OJIP measurements were calculated according to Guo et al. (2020).

Data analysis

Analysis of variance (ANOVA) was performed using SAS9.1. The significance of differences between treated groups and the control was analyzed using the unequal variance two-tailed Student's t-test, and significant differences among ATZ concentrations was determined by Duncan's test, and P value <0.05 was considered significant.

Results

Chlorophyll fluorescence rise kinetics OJIP curves

For the treatments with ATZ only, the level of J-step of fluorescence rise kinetics increased for all ATZ concentrations (Fig. 1A); the I- and P-step levels also increased at ATZ concentrations of 0.1, 0.5, and 1.0 $\text{mg}\cdot\text{L}^{-1}$, but declined at ATZ concentrations of 2.0 $\text{mg}\cdot\text{L}^{-1}$. ATZ at concentrations of 1.0 and 2.0 $\text{mg}\cdot\text{L}^{-1}$ led to the disappearance of J-I and I-P phases. In addition, the uptrend in F_o and downtrend in F_v/F_o with the increase of ATZ concentration were observed, and the change trend showed a significant dose effect relationship (Fig. 1B). F_m decreased significantly in treatment with 2.0 $\text{mg}\cdot\text{L}^{-1}$ ATZ. It can be seen that the decrease in F_v/F_o was mainly attributable to the rise of F_o when ATZ concentrations were 0.5 and 1.0 $\text{mg}\cdot\text{L}^{-1}$; while it was associated not only with the increase of F_o but also with the decrease of F_m when ATZ concentration was 2.0 $\text{mg}\cdot\text{L}^{-1}$. ATZ alone at 0.1 $\text{mg}\cdot\text{L}^{-1}$ did not differ significantly from the control in F_o , F_m and F_v/F_o .

For treatments with Cd alone, Cd at concentrations of 5 $\text{mg}\cdot\text{L}^{-1}$ caused slight increases in J-, I- and P-step with obvious J-I and I-P phases (Fig. 1C), F_o , F_m and F_v/F_o did not change significantly compared with the control (Fig. 1D); When Cd concentration increased to 10 $\text{mg}\cdot\text{L}^{-1}$, however, the J-step level increased while the P-step level decreased (Fig. 1E), F_m and F_v/F_o were significantly lower than those of the control (Fig. 1F).

For treatments with ATZ plus Cd, OJIP curves showed visible J-, I- and P-step with lower F_o and higher F_v/F_o in comparison with ATZ alone (Fig. 1C, E); the levels of J-, I- and P-step were higher and the magnitude of J-I phase was still lower than those of the control except for 0.1 $\text{mg}\cdot\text{L}^{-1}$ ATZ combined with 5 $\text{mg}\cdot\text{L}^{-1}$ Cd; when ATZ concentrations were 1.0 and 2.0 $\text{mg}\cdot\text{L}^{-1}$, F_o and F_m increased significantly, and F_v/F_o did not showed significant compared with those of the control.

The double normalized fluorescence curves (presented as relative variable fluorescence V_t on a logarithmic time scale) show invisible features of OJIP curve (Guo et al. 2020). Compared with the control, J-step increased drastically under ATZ stress whether in presence or absence of Cd, and ATZ alone induced a greater increase than ATZ combined Cd did (Fig. 2A, C, E). Especially in the case of ATZ alone at concentrations above 0.1 $\text{mg}\cdot\text{L}^{-1}$, J-step was closed to P-step with a disappearance of the IP phase of the OJIP curve; at the same time, a remarkable lift in I-step and a flattened I-step was observed (Fig. 2A). The initial slope of the relative variable fluorescence kinetics, denoted as M_0 , expresses the net rate of the RCs' closure (Strasser et al. 2004). The M_0 values of single and combined treatments of ATZ and Cd were significantly larger than that of the control (Fig. 2A, C, E; Table 2). ATZ alone at concentrations above 0.1 $\text{mg}\cdot\text{L}^{-1}$ had stronger effects on the M_0 value than ATZ combined with Cd did; and the difference was significant when Cd concentration was 10 $\text{mg}\cdot\text{L}^{-1}$. The ANOVA result showed that ATZ and Cd were significant factors for the M_0 value and had statistically significant interaction effects (Table 2). V_J , V_I and F_J/F_I further reflected the initial action site of ATZ. V_J and V_I reflect the accumulation of Q_A^- (Sun et al. 2020). Both single ATZ and ATZ combined Cd increased significantly the values of V_J , V_I

and F_J/F_I compared with the control. ATZ only at levels above $0.1 \text{ mg}\cdot\text{L}^{-1}$ showed significant differences in values of V_I , V_I and F_J/F_I from ATZ combined with $10 \text{ mg}\cdot\text{L}^{-1}$ Cd, whereas this difference was not observed when Cd concentration was $5 \text{ mg}\cdot\text{L}^{-1}$ (Fig. 2B, D, F). Under single ATZ and combined stresses of ATZ and Cd, V_J showed higher increase magnitude than V_I .

The initial slope of the standardized fluorescence transient F_t/F_o

To ascertain the differences in the rate of the electron transfer from P_{680} to Q_A between ATZ-Cd treated and control plants, the initial slopes of the standardized fluorescence transient (the fluorescence values were expressed as F_t/F_o (Strasser et al. 2004)) at a linear time scale from 0.02 to 0.15 ms were presented in Fig. 3. The initial slope of single ATZ at the level of 0.1 and $0.5 \text{ mg}\cdot\text{L}^{-1}$ was significantly greater than that of the control, while it markedly decreased when ATZ concentration was up to $2.0 \text{ mg}\cdot\text{L}^{-1}$ (Fig. 3A, E). In contrast to the control, the plant treated with single Cd showed a significant increase in the initial slope, and there was significant difference among two Cd concentrations (Fig. 3B, C, E). The initial slope of ATZ combined with Cd was significantly larger than that of the control, and was significantly larger than that of ATZ alone when ATZ concentrations were 1.0 and $2.0 \text{ mg}\cdot\text{L}^{-1}$.

L-band

L-band is known as an indicator of the grouping of the PSII units or energetic connectivity between antenna and PSII RCs (Guo et al. 2020). For single ATZ treatment, the L-band increased with increasing ATZ concentrations. ATZ at the concentration of 1.0 and $2.0 \text{ mg}\cdot\text{L}^{-1}$ increased L-band most obviously (Fig. 4A) with significantly higher W_L and F_L/F_J (Fig. 4B); ATZ at other concentrations yielded negative L-band values, and the levels of W_L and F_L/F_J for $0.1 \text{ mg}\cdot\text{L}^{-1}$ ATZ were significantly different from those of the control. For the treatments with Cd alone, L-band slightly increased (Fig. 4C, E), and there was no significant difference in W_L and F_L/F_J compared with the control (Fig. 4D, F). ATZ combined with Cd, except for $0.1 \text{ mg}\cdot\text{L}^{-1}$ ATZ combined with $5 \text{ mg}\cdot\text{L}^{-1}$ Cd, decreased L-band (Fig. 4C, E) with significantly smaller W_L and F_L/F_J compared with the control (Fig. 4D, F). In addition, when ATZ concentrations were 1.0 and $2.0 \text{ mg}\cdot\text{L}^{-1}$, the W_L and F_L/F_J values for ATZ alone were significantly larger than those of ATZ combined with Cd.

K-band

The inhibition of the donor side of PSII results in the appearance of K-step in the chlorophyll *a* fluorescence rise (Lazár 2006). The double normalized chlorophyll *a* fluorescence curve in the 0.02–2 ms time range revealed the changes of K-band (Fig. 5). *I. pseudacorus* exhibited a prominent rise of fluorescence intensity at K points of the transient curve in response to ATZ alone at concentrations of 1.0 and $2.0 \text{ mg}\cdot\text{L}^{-1}$ (Fig. 5A), and the values of W_K and F_K/F_J were significantly higher than those of the control (Fig. 5B). The exception was the treatments with ATZ alone at $0.1 \text{ mg}\cdot\text{L}^{-1}$ which showed a negative K-band with a significantly decreased F_K/F_J (Fig. 5A, B), but this significant difference was not

observed in W_K (Fig. 5B). Compared with the control, Cd alone induced the occurrence of K-band, and only Cd at $10 \text{ mg}\cdot\text{L}^{-1}$ increased significantly the values of W_K and F_K/F_J . It was observed that the K-band was negative for $2.0 \text{ mg}\cdot\text{L}^{-1}$ ATZ combined with Cd (Fig. 5C, E), and F_K/F_J decreased significantly compared to that of the control, while W_K did not increase pronouncedly (Fig. 5D, F). On the other hand, when ATZ concentrations were 1.0 and $2.0 \text{ mg}\cdot\text{L}^{-1}$, the F_K/F_J values for ATZ alone were significantly larger than those of ATZ combined with Cd, but W_K did not show significant changes.

Energy conservation and photosynthetic performance

In the JIP-test parameters, the absorbed light energy (ABS/RC), captured light energy (TR_0/RC), thermally dissipated light energy (DI_0/RC), and the energy used for electron transfer (ET_0/RC) on the unit of active center, were used to indicate the energy transformation in the PSII (Liu et al. 2020). For the treatment with single ATZ, there was no significant difference in ABS/RC (Fig. 6A) and DI_0/RC (Fig. 6D) between ATZ at levels below $0.5 \text{ mg}\cdot\text{L}^{-1}$ and the control, while ATZ at levels of 1.0 and $2.0 \text{ mg}\cdot\text{L}^{-1}$ increased significantly ABS/RC and DI_0/RC ; ATZ at the level of $1.0 \text{ mg}\cdot\text{L}^{-1}$ also caused a significant increase in TR_0/RC (Fig. 6B); ET_0/RC decreased significantly at all ATZ concentrations (Fig. 6C). For the treatment with single Cd, ABS/RC , TR_0/RC and DI_0/RC increased significantly, while ET_0/RC did not differ from the control. For the combined treatment with ATZ and Cd, ABS/RC and DI_0/RC at high level ATZ (1.0 and $2.0 \text{ mg}\cdot\text{L}^{-1}$) were significantly lower than those of corresponding single ATZ treatments; ET_0/RC of 1.0 and $2.0 \text{ mg}\cdot\text{L}^{-1}$ ATZ was significantly higher than that of ATZ alone when Cd concentration was $10 \text{ mg}\cdot\text{L}^{-1}$, but this significant difference was not observed when Cd concentration was $5 \text{ mg}\cdot\text{L}^{-1}$.

PI_{abs} is the performance index for energy conservation from exciton to the reduction of intersystem electron acceptors, and PI_{total} is performance index for energy conservation from exciton to the reduction of PSI end acceptors (de Souza et al. 2020). For the treatment of single ATZ or single Cd, PI_{abs} (Fig. 7A) and PI_{total} (Fig. 7B) declined significantly compare with the control. For the combined treatment with ATZ and Cd, the values of PI_{abs} and PI_{total} were greater than those of the corresponding single ATZ treatment when ATZ concentrations were at levels 1.0 and $2.0 \text{ mg}\cdot\text{L}^{-1}$.

Discussion

In the present study, the individual and combined effect of ATZ and Cd on photosystems behavior in *I. pseudacorus* leaves was evaluated using the fluorescence rise OJIP kinetics. The prompt fluorescence kinetics based on the energy cascade is useful for quantifying variations in the donor and acceptor sides of PS II, and is now widely employed to investigate electron transport in plant leaves because of its fast, non-invasive, precision and inexpensive characteristics (Guo et al. 2020; Sun et al. 2020). A typical Chlorophyll *a* fluorescence curve has four basic steps: O ($20 \mu\text{s}$) refers to the initial fluorescence level (F_0), J (2 ms) and I (30 ms) are intermediate level, and P is the peak level (F_m) (Gao et al. 2018; Guo et al. 2020). In the OJIP curve, the O-J phase represents the reduction of the acceptor side of PSII, the J-I phase

represents the reduction of the PQ pool, and the I-P phase reflects the reduction of the acceptor side of PSI (Ji et al. 2018).

The herbicide ATZ, as a PSII inhibitor, mainly lies in the fact that it can bind to the Q_B -binding niche on the D1 protein of PSII, thus blocks the photosynthetic electron transport at PS II and excitation energy transfer from PS II to PS I (Majewska et al. 2018). In the present work, both single ATZ and ATZ combined Cd caused a significant increase in F_o and a dramatic decline in F_v/F_o except for ATZ concentration of $0.1 \text{ mg}\cdot\text{L}^{-1}$. F_o fluorescence mainly depends on the initial exciton density in the PSII antenna pigment and, the structure state of the excitation energy transfer between the antenna pigments and the antenna pigment to the RCII (Krause and Weis 1984). Higher F_o is symptomatic of a stronger overall emission which derived from functional changes within photosynthetic membranes (Kriedemann et al. 1985), corresponding to energy losses in the pigments of the PSII antenna and less efficient energy transfer among the antennae complexes toward the RCII (Kalaji et al. 2018), and subsequently causing a decrease in the rate constant of energy trapped by RCII (Hassannejad et al. 2020). Therefore, this increase in F_o was mainly for two reasons: (1) the increase of initial exciton density resulted from the blocking of electron transport beyond Q_A ; and (2) the low connectivity of PSII units caused low energy transfer from LHCII to RCII and thus higher fluorescence from LHCII (Tovuu et al. 2013; Ghassemi-Golezani and Lotfi 2015). The F_v/F_o , responding very sensitively to any changes in F_v and/or F_o , is also used to evaluate the photosynthetic quantum conversion efficiencies in dark-adapted (Babani and Lichtenthaler 1996; Govindachary et al. 2004). The progressive and significant decrease in F_v/F_o with increasing ATZ concentrations indicated that the water-splitting system of the donor side of PSII might be seriously impacted by high concentration ATZ (Mosadegh et al. 2019). F_m could reflect the status of the electronic pass through the PSII. It primarily depends on the amount of Q_A^- (Singh-Rawal et al. 2010), and is high when the Q_B binding site is occupied by a PSII herbicide (Prášil et al. 2018). In addition, increasing F_m is associated with the rise of PSII activity due to conformational changes in the D1 protein (Ghassemi-Golezani and Lotfi 2015). In this study, an increasing trend in F_m of treatments of single ATZ at concentrations of 0.1 and $0.5 \text{ mg}\cdot\text{L}^{-1}$ illustrated better physiological regulation ability and adaptability of *I. pseudacorus* to ATZ stress at this level. However, a significant decrease in F_m was observed when ATZ concentration reached $2 \text{ mg}\cdot\text{L}^{-1}$, suggesting that ATZ inhibited electron donation from OEC to P_{680}^+ (Singh-Rawal et al. 2010), and OEC was inactivated in *I. pseudacorus* under this condition (Mathur et al., 2011). In addition, this decrease also indicated that the reduction of both the PQ pool and the electron transport acceptors around PSI was inhibited (Martins et al. 2018). A gradually significant decline in the ratio F_v/F_o of treatments with ATZ alone above $0.1 \text{ mg}\cdot\text{L}^{-1}$ displayed that the potential photochemical efficiency of PSII decreased with increasing ATZ concentrations, which was associated with a disruption of water-splitting system (Borkowska 2002). Furthermore, significant changes in F_v/F_o between ATZ concentrations indicated that F_v/F_o was a sensitive indicator of ATZ induced limitation of photosynthesis in *I. pseudacorus*.

In this study, the relative variable fluorescence induction kinetic curves on a logarithmic time scale showed that J-I and I-P phases disappeared due to a sharp increase of J- and I-step closed to the same

level as P-step in the treatments with ATZ alone at levels above $0.1 \text{ mg}\cdot\text{L}^{-1}$. These results implied that, under single ATZ stress, Q_A was reduced entirely and the electron flow from Q_A^- to Q_B was blocked (Chen et al. 2007). Additionally, the loss of I-step from chlorophyll fluorescence induction curves can be linked to the inactivation of PSI and suppression of the cyclic phosphorylation (Cetner et al. 2020). The increased value of V_J and V_I parameters suggests the accumulation of reduced Q_A and PQ, which cannot transfer electrons to the dark reactions (Kalaji et al. 2014). This point was proven matching very well with the decrease in ET_0/RC . Here, the values of V_J and V_I increased, suggesting a decline in the exchange capacity of PQs at the Q_B site and reoxidation capacity of plastoquinol (PQH_2). The reoxidation of PQH_2 is related to the activity of PSI, whereas the exchange of PQs at the Q_B site depends on electrons coming from PSII (Gao et al. 2014). Therefore, the more obvious increase degree of V_J and V_I indicated that the activities of PSI and PSII were susceptible to ATZ. It was noticeable that the remarked increase of V_J and F_J/F_I was mainly attributed to the increase in F_J further confirming that PSII electron transport rate decreased in *I. pseudacorus* (Singh-Rawal et al. 2010). A sharp rise of J-step contributed to the large accumulation of Q_A^- , causing an increase in the net rate of the RCs' closure. As indicated by the larger values of M_0 in treatments of single ATZ relative to the control, also proved that ATZ interrupted PSII electron transport beyond Q_A (Guo et al. 2020).

The appearance of the K-step is due to the accumulation of Y_Z^+ (a tyrosine residue in the D1 subunit), which results from an imbalance within PSII between the electrons leaving the RC at the acceptor side and the electrons donated by the donor side, and is widely used to represent the state of the active OEC centers at the PSII donor side (Strasser 1997; Strasser et al. 2004). W_K and the ratio F_K/F_J reflect the changes in the amplitude in the K-step (Strasser 1997; Lu and Zhang 2000). The considerable increase in W_K and F_K/F_J was observed in the treatments with single ATZ at or above $1.0 \text{ mg}\cdot\text{L}^{-1}$, indicating a noticeable imbalance between electron flow from the OEC to Y_Z and electron flow from P_{680} to Q_A and beyond (Strasser 1997), and the inactivation of the PSII donor side (Mosadegh et al. 2019). The L-band can give information on the energy connectivity or grouping among PS II units. A positive L-band indicates the lower energetic connectivity while a negative L-band is a sign of greater grouping and more efficient energy exchange between the neighboring PSII units (Kalaji et al. 2018; Martins et al. 2018). ATZ alone at higher levels ($\geq 1.0 \text{ mg}\cdot\text{L}^{-1}$) caused a prominent lift in L-band with the significant rise in the values of W_L and F_L/F_J indicating a decrease in energetic connectivity among PSII units. However, *I. pseudacorus* grown in a medium with ATZ alone of 0.1 showed a negative L-band with significantly decreased W_L and F_L/F_J , implicating higher connectivity of antennae to RCII units and more efficient utilization of excitation energy (Mlinarić et al. 2017). This corresponded well with the increase in the initial slope of Ft/Fo curve. These results suggested that this plant species exhibited relatively high tolerance to ATZ stress at this level through enhancing the electron supply from P_{680} to Q_A and energetic connectivity. To minimize damage to the photosynthetic apparatus, excess energy can be dissipated as nonphotochemical processes, including thermal dissipation (Silva et al. 2021). In this study, the gradual increase in DI_0/RC with increasing ATZ concentration suggested that partial excitation energy was

dissipated in nonphotochemical processes, especially achieving significant differences at higher levels of ATZ ($\geq 1.0 \text{ mg}\cdot\text{L}^{-1}$). The significant increase in the nonphotochemical quenching was also observed by Silva et al. (2021) in determining the sensitivity level of *Handroanthus heptaphyllus* to ATZ. Though this increase diminished the fraction of energy delivered to PS II centers, it played an important role in the mitigation against photodamage, which reflected the tolerance of *I. pseudacorus* to ATZ by activating the photoprotective responses (Cazzaniga et al. 2013).

Though the target action site of ATZ was located on the electron acceptor side in PSII, some indirect effects, such as the inactivation of OEC and PSI, can be seen from the appearance of K-step and the disappearance of I-step, respectively. Therefore, ATZ eventually caused the overall suppression of plant photosynthesis owing to the less grouping of the PSII units and the imbalance of the electron flow (Sun et al. 2020). Besides, the overall impact of ATZ on the photosynthesis was likely linked to the oxidative stress in plants trigger by blocking of photosynthetic electron transport (Zhang et al. 2014; Guo et al. 2020).

Compared with ATZ alone, ATZ combined with Cd presented lower F_o and higher F_v/F_o , which meant less light energy dissipation in antenna and smaller structural alterations on the donor side of PSII (Mathur et al. 2011). This was consistent with what was indicated clearly by the higher ET_0/RC and lower DI_0/RC . The fluorescence rise transient obtained from the treatments of ATZ combined with Cd, especially at high ATZ concentration ($\geq 1.0 \text{ mg}\cdot\text{L}^{-1}$), showed an apparent I-P phase, and a low fluorescence intensity at K point relative to ATZ alone, as well as negative L-band values, suggesting that the coexistence of Cd conduced to maintain the balance of electron transfer from the donor side to the acceptor side of PSII, and was helpful for the maintenance of the energetic connectivity and efficient consumption of the excitation energy and stability of the system upon ATZ treatments. It was noteworthy that no significant effect of single ATZ at $2 \text{ mg}\cdot\text{L}^{-1}$ on W_K meant that the significant decrease in F_K/F_J was attributed mainly to the decrease of the J-step level, suggesting that the presence of Cd mainly reduced the damage to the acceptor side in this situation. This coincided exactly with the action mechanism of ATZ by which ATZ displaced the secondary quinone acceptor, Q_B , from its binding site at the D1 protein of PSII. ATZ stress-induced damage to the structure and function of PSII and PSI in leaves of *I. pseudacorus* was alleviated by the presence of Cd, as indicated by the significantly higher values of PI_{abs} and PI_{total} in combined treatments relative to single ATZ. The inference was also supported by the initial slope of the standardized fluorescence transient, an indication of the electron transfer rate from P_{680} to Q_A , which was higher in combined treatments relative to the corresponding single ATZ treatments, suggesting that the electron transfer rate in PSII was enhanced in the presence of Cd. These results were quite matched with a former report, which showed that ATZ in binary mixtures with Cd was less toxic than ATZ alone to rice seedlings (Su et al. 2005). The enhanced photosynthetic performance in the presence of Cd may be related to the formation of the complex between ATZ and Cd^{2+} . ATZ contains one or more binding sites (most probably NH) and they can potentially participate in the formation of the metal ion-ATZ dimer complexes (Su et al. 2005; Singh et al. 2020). A previous study confirmed that Cd can potentially complex with either monomeric or dimeric ATZ to form both anhydrous and hydrated complex forms (Meng and

Carper 2000). Moreover, the N atoms of ATZ have stronger complexation ability with Cd^{2+} than with other divalent metal ions, such as Zn^{2+} and Cu^{2+} (Martin et al. 1998). Since Cd^{2+} occupied the binding site of ATZ with D1 protein, the competitive binding to the Q_B -niche of the D1 protein was limited, contributing to slighter inhibitory effects of ATZ on *I. pseudacorus*. For most of the selected JIP-test parameters, there were no statistically significant differences between the two Cd concentrations selected in this study. This was probably associated with the concentration ratio of ATZ to Cd. A former research indicated that at the 1:1 molar concentration ratio, the formation of the complex between ATZ and Cd notably reduced the individual toxicities to rice seedlings (Su et al. 2005). However, the ratios in our study, ranging from 0.005 to 0.2, were far less than 1. Therefore, for better understanding of the toxicity of mixture of ATZ and Cd to plants, the ratio ranges both above and below 1 should be applied in the future study. In addition, the lower magnitude of the J-P phase in comparison with the control indicated that the PSII donor side was still partially inhibited by ATZ combined with Cd. Also, the electron transport flux and energy conservation capacity of the photosynthetic apparatus decreased, seen as the lower ET_0/RC , as well as the significant decrease in PI_{abs} and PI_{total} relative to the control. Therefore, ATZ combined with Cd still had inhibitory effects on photosynthetic performance of *I. pseudacorus*. Apart from ATZ, Cd might also be responsible for these adverse changes in photosynthetic characteristics in *I. pseudacorus* leaves, as can be seen from the positive K-band and significant decreases in PI_{abs} and PI_{total} caused by Cd alone. Other studies also indicated that Cd inhibited both the donor and the acceptor site of PSII and, thus, reduced the photosynthetic capacity of plants (Sigfridsson et al. 2004; Kalaji et al. 2016; Huihui et al. 2020).

Conclusion

ATZ showed the similar inhibitory activity on electrons transfer from Q_A to Q_B , mainly increasing the J-step on prompt fluorescence curves of non-target aquatic plant species, *I. pseudacorus*. It also had indirect impacts on the donor side because of the imbalance of electron in and out of RCs and subsequently the less efficient energy exchange between the neighboring PSII units. *I. pseudacorus* exhibited tolerance to ATZ at concentrations not above $0.5 \text{ mg}\cdot\text{L}^{-1}$ by increasing the rate of the electron transfer from P_{680} to Q_A and energetic connectivity between PSII units, but was sensitive to ATZ at higher levels ($\geq 1 \text{ mg}\cdot\text{L}^{-1}$). Due to the formation of ATZ-Cd complex, ATZ mixed with Cd displayed weaker influence on the photosynthesis ability relative to ATZ alone, whereas still had harmful effects on the photosynthetic apparatus of *I. pseudacorus*. These results imply that the potential ecological risk of combined pollution of ATZ and Cd in waters still exist and cannot be ignored, even though lower than that of ATZ alone.

Abbreviations

PSII – photosystem II;

PSI – photosystem I;

F_o – initial fluorescence;

F_m – maximum fluorescence;

F_v – variable fluorescence defined as F_m – F_o;

LHCII – light-harvesting complexes II;

RCII – reaction center of PSII;

PQ – plastoquinone;

Q_A and Q_B – primary and secondary plastoquinone acceptors of PS II, respectively;

OEC – oxygen evolution complex;

Y_Z – a tyrosine residue in the D1 subunit.

Declarations

Authors' contributions All authors contributed significantly to the preparation of this manuscript. Qinghai Wang and Xiaoe Que designed the research, Dongyu Xie and Lei Peng performed experiments, Qinghai Wang and Dongyu Xie analyzed data and drafted the manuscript, Cui Li performed plant cultivation and management, Chuansheng Chen supported methodology. All authors read the manuscript and approved the final manuscript.

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Consent to publish All authors declare (i) that this is the original research article which in full or any part whatsoever, has not been published, accepted for publication or under editorial review for publication elsewhere; and that our institutes' representative "Beijing Academy of Agriculture and Forestry Sciences, Beijing, China; Central South University of Forestry and Technology, Changsha, China; and Chinese Academy of Forestry, Beijing, China" is fully aware of this submission. (ii) Upon acceptance for publication authors confirm and grant the Environmental Science and Pollution Research (ESPR) journal the right to publish this article in accordance with the journal's copyright policy.

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Tables

Table 1 The selected JIP-test parameters used in this study

Technical fluorescence parameters	
$F_o \approx F_{0.02\text{ms}}$	Fluorescence intensity at 0.02 ms of OJIP
$F_K = F_{0.3\text{ms}}$	Fluorescence intensity at 0.3 ms of OJIP
$F_J = F_{2\text{ms}}$	Fluorescence intensity at the J-step (2 ms) of OJIP
$F_I = F_{30\text{ms}}$	Fluorescence intensity at the I-step (30 ms) of OJIP
$F_m = F_P$	Maximal recorded fluorescence intensity, at the peak P of OJIP
$F_v = F_m - F_o$	Maximal variable fluorescence
$V_t = (F_t - F_o) / (F_m - F_o)$	The relative variable fluorescence at time t
$V_J = (F_J - F_o) / (F_m - F_o)$	The relative variable fluorescence at 2 ms
$V_I = (F_I - F_o) / (F_m - F_o)$	The relative variable fluorescence at 30 ms
$W_{OK} = (F_t - F_o) / (F_K - F_o)$	The ratio of variable fluorescence used to clarify L-band
$W_{OJ} = (F_t - F_o) / (F_J - F_o)$	The ratio of variable fluorescence used to clarify K-band
$M_0 = 4(F_{0.27\text{ms}} - F_o) / (F_m - F_o)$	The approximated initial slope (in ms^{-1}) of the fluorescence transient normalized on the maximal variable fluorescence F_v
ABS/RC, TR ₀ /RC, DI ₀ /RC and ET ₀ /RC	The absorbed, captured, thermally dissipated light energy, and the energy used for electron transfer per reaction center, respectively
PI _{abs} and PI _{total}	The performance index for energy conservation from exciton to the reduction of intersystem electron acceptors, and the performance index for energy conservation from exciton to the reduction of PSI end acceptors, respectively

Table 2 The initial slope (calculated as $M_0 = (\Delta V / \Delta t)_0 = (V_{0.27\text{ms}}) / (0.25 \text{ ms})$) of the relative variable fluorescence on a linear timescale from 0.02 to 0.3 ms

Cd concentrations (mg·L ⁻¹)	ATZ concentrations (mg·L ⁻¹)				
	0	0.1	0.5	1.0	2.0
0	0.75±0.14	1.27±0.25 a **	1.95±0.38a **	2.42±0.34 a **	2.57±0.21 a **
5	0.93±0.08 *	1.31±0.34 a **	1.78±0.27a **	1.88±0.25 b **	1.81±0.27 b **
10	1.46±0.22 **	1.17±0.14 a **	1.28±0.21b **	1.59±0.29 b **	1.32±0.23 c **
Analysis of variance					
	DF	SS	MS	F Value	<i>P</i>
ATZ	4	9.36	2.34	37.42	<0.0001
Cd	2	2.12	1.06	16.95	<0.0001
ATZ×Cd	8	5.73	0.72	11.47	<0.0001

Note: Different letters in the same column indicate significant differences ($P<0.05$); * and ** represent significant difference between treatments and the control at the levels of 0.05 and 0.01, respectively.

Figures

Figure 1

Raw chlorophyll a fluorescence OJIP transient curves, and values of F_o , F_m and F_v/F_o in *I. pseudacorus* treated with ATZ alone (A, B), treated with ATZ plus 5 mg·L⁻¹ Cd (C, D), and treated with ATZ plus 10 mg·L⁻¹ Cd (E, F). Each parameter followed by different letters show significant differences at $P < 0.05$ among different treatments according to the Duncan's test.

Figure 2

The relative variable fluorescence induction kinetic curves on a logarithmic time scale, and the values of V_J , V_I and F_J/F_I in *I. pseudacorus* treated with ATZ alone (A, B), treated with ATZ plus 5 mg·L⁻¹ Cd (C, D), and treated with ATZ plus 10 mg·L⁻¹ Cd (E, F). The insert figure presents the relative variable fluorescence on a linear timescale from 0.02 to 0.3 ms. Each parameter followed by different small case letters are significantly different ($P < 0.05$) among different treatments according to the Duncan's test.

Figure 3

The OJIP transients normalized by F_0 as F_t/F_0 on a linear time scale from 0.02 to 0.15 ms in *L. pseudocorus* treated with ATZ alone (A), treated with ATZ plus 5 mg·L⁻¹ Cd (B), and treated with ATZ plus 10 mg·L⁻¹ Cd (C), and values of the initial slope (D). Different small case letters for same ATZ concentrations indicate significant differences (P < 0.05) between Cd treatments according to the Duncan's test, and different upper-case letters indicate significant differences (P < 0.05) between single ATZ treatments according to the Duncan's test.

Figure 4

The fluorescence rise kinetics normalized by FO and FK as WOK on a linear time scale from 0.02 to 0.3 ms, and the values of W_L and F_L/F_J in *I. pseudacorus* treated with ATZ alone (A, B), treated with ATZ plus 5 mg·L⁻¹ Cd (C, D), and treated with ATZ plus 10 mg·L⁻¹ Cd (E, F). Each parameter followed by different small case letters are significantly different ($P < 0.05$) among different treatments according to the Duncan's test.

Figure 5

The fluorescence rise kinetics normalized by FO and FJ as WOJ on a linear time scale from 0.02 to 2 ms, and the values of WK and FK/FJ in *I. pseudacorus* treated with ATZ alone (A, B), treated with ATZ plus 5 mg·L⁻¹ Cd (C, D), and treated with ATZ plus 10 mg·L⁻¹ Cd (E, F). Each parameter followed by different small case letters are significantly different ($P < 0.05$) among different treatments according to the Duncan's test.

Figure 6

Specific energy fluxes per reaction center in *I. pseudacorus* treated with ATZ and Cd: (A) ABS/RC, (B) TR₀/RC, (C) ET₀/RC, and (D) DI₀/RC. Different small case letters for same ATZ concentrations indicate significant differences ($P < 0.05$) between Cd treatments according to the Duncan's test, and different upper-case letters indicate significant differences ($P < 0.05$) between single ATZ treatments according to the Duncan's test.

Figure 7

Photosynthetic performance indexes, PIABS (A) and PI_{total} (B), of *I. pseudacorus* treated with ATZ and Cd. The data are mean $\pm$ SD. Different small case letters for same ATZ concentrations indicate significant differences ($P < 0.05$) between Cd treatments according to the Duncan's test.