Effects of ciprofloxacin on turion germination and seedling development in two submerged aquatic plants

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Abstract

Germination and seedling development are crucial processes for plant growth and survival, and asexual propagules are predominant reproduction organs for aquatic plants. Ciprofloxacin is widely detected in both terrestrial and aquatic ecosystems, while studies on its effects on germination and seedling development mainly focused on terrestrial plants. We evaluated effects of ciprofloxacin (0.1, 1, 5 and 10 mg/L) on turion germination and early establishment of two submerged plants species (*Potamogeton crispus* and *Hydrilla verticillata*). Results showed that germination rate and rooting rate of both species were not impacted by ciprofloxacin. However, 0.1 mg/L ciprofloxacin significantly accelerated germination of *H. verticillata* and 5-10 mg/L ciprofloxacin significantly delayed rooting of both species. With rising ciprofloxacin concentrations and prolonging exposure time, seedling tolerance index and root number of both species decreased significantly, and shoot number decreased slightly in *P. crispus* but kept increasing in *H. verticillata*, suggesting better tolerance of *H. verticillata* under ciprofloxacin exposure. Root and shoot biomass accumulation, photosynthetic pigments contents of both species were all significantly inhibited. To defense against oxidative stress and prevent lipid peroxidation, antioxidants of both species including superoxide dismutase, catalase, guaiacol peroxidase, polyphenol oxidase, ascorbate peroxidase, ascorbic acid, reduced glutathione were activated to varying degrees. By integrating biomarker responses, we found the stress degree of ciprofloxacin on seedlings of both species was enhanced with increasing ciprofloxacin concentrations. Overall, ciprofloxacin impacted turion germination process and harmed early establishment of both species, suggesting potentially adverse effects of ciprofloxacin pollution on survival and expansion of submerged aquatic plant populations.

1. Introduction

Submerged plants, the main primary producers in shallow fresh waters, could provide habitats, shelters and food sources for fishes, aquatic macroinvertebrates and waterbirds, contributing to their important roles in the maintenance of structure and function of shallow-water ecosystems (Yuan et al. 2018; Özgencil et al. 2020). However, in recent years, because of factors such as climate changes (Yan et al. 2021), fishery impacts (Ren et al. 2022) and water pollutions (Wu et al. 2021; Hua et al. 2022; Zhang et al. 2021), submerged vegetations are experiencing serious deterioration (Phillips et al. 2016). For instance, in eutrophic water bodies, toxic microcystins produced by massive cyanobacterial blooms would deteriorate water quality and seriously threaten the growth and survival of submerged plants (Ha and Pflugmacher 2013). Additionally, as emerging pollutants, fluoroquinolone antibiotics have become one of the most common categories of harmful pollutants in lakes (Chen et al. 2020), rivers (Pan et al. 2020) and even seawaters (Wu et al. 2022). Polluted waters containing fluoroquinolone antibiotics could impact aquatic community structure and might act as possible causes of the decline and disappearance of aquatic plants (Robinson et al. 2005).

Ciprofloxacin is a fluoroquinolone antibiotic that has been widely used in aquaculture, treatments of human ailments and pharmaceutical industries. Consequently, agricultural wastewater, hospital effluent, domestic sewage and industrial emissions are the main sources of ciprofloxacin pollution in natural water bodies (Adeleye et al. 2022). For example, ciprofloxacin (mean concentration, detected frequency) was detected in

drinking water (169.2 ng/L, 94.9%), surface water (28 ng/L, 100%) and ground water (77.2 ng/L, 45%) (Boy-Roura et al. 2018; Pan et al. 2020; Chen et al. 2018). Enormously high concentrations of ciprofloxacin in effluents of wastewater treatment plants (14 mg/L) and lakes (6.5 mg/L) have also been reported by Fick et al (2009). In estuarine water and seawater of Laizhou Bay in northern China, ciprofloxacin was one of the most dominant antibiotics which contributed to over 70% of the total antibiotic burden (Lu et al. 2022). Moreover, by inducing antibiotic resistance (Yang et al. 2020), ciprofloxacin could cause adverse effects on human health at even low concentrations (ng/L) (Koczura et al. 2012; Marchant 2018). Therefore, ciprofloxacin has been proposed to be prioritized and strictly controlled to reduce its negative effects on food chains and ecological systems (Han et al. 2020).

Generally, environmental stresses could impact vegetative growth and physiological stability of aquatic plants (Bornette and Puijalon 2010; Yu et al. 2022). For example, ciprofloxacin (0.01-1mg/L) significantly decreased the photosynthetic pigment contents, disrupted PSII integrity, affected leave growth and triggered reactive oxygen species (ROS) accumulations and oxidative stress in a floating plant *Eichhornia crassipes* (Yan et al. 2019). Root developments, chlorophyll contents, superoxide dismutase (SOD) and guaiacol peroxidase (POD) activities and root activity of the emergent plant species *Phragmites australis* were all evidently inhibited by a mixture of ciprofloxacin, oxytetracycline and sulfamethazine (Liu et al. 2013). As for submerged plants, Ebert et al (2011) found the 7-day NOEC (the no-observed-effect concentration) of ciprofloxacin based on root elongation of *Myriophyllum spicatum* L was higher than 0.98 mg/L. These studies mainly focused on vegetative growth stage. However, researches on other life history stages such as germination and reproduction are limited. As an example, it has been reported that ciprofloxacin at 0.2-2 mg/L did not affect the germination rate of maize seeds but significantly reduced the germination time (Gomes et al. 2019). But no investigations on the effects of ciprofloxacin on germination phase of aquatic species have been reported.

Vegetative propagation is one of the main modes of reproduction for submerged plants, and turions of submerged plants are modified vegetative organs produced via asexual processes (Adamec 2018). With functional similarity to seeds, turions also have the ability to generate progeny plants (Song et al. 2017). What is more, due to their tolerance to extreme conditions such as coldness, darkness and hypoxiation of the underwater environments, turions play key roles in maintaining submerged plant community (Adamec 2008). Nowadays, in China, sowing turions of submerged plants have become one of the most common practices for restoration of damaged submerged vegetation in lakes and ponds (Jian et al. 2003; Song et al. 2017). Undoubtedly, germination of turions and early establishment are crucial stages that could directly influence the biomass accumulations and population expansion of submerged plants. *Hydrilla verticillata* is a dominant submerged species in many freshwater lakes, while *Potamogeton crispus* could be widely found in shallow lakes, ponds, rice paddies and rivers (Jian et al. 2003; Li et al. 2021). Additionally, due to their good performances in purifying water, these two submerged plants have been widely used as pioneer species in restoration of submerged plants for governing eutrophication (Li et al. 2021; Wang et al. 2017). However, ecotoxicological effects of ciprofloxacin on turion germination and seedling development of these two submerged plants have not been reported.

Biomarkers, or biochemical endpoints, are often used to clarify the mechanism of action of chemicals. Moreover, there usually more than one biomarker response is assessed in organisms exposed to toxic chemicals or stressed conditions. Thus, integrating multiple biomarkers is a valid and reliable approach to characterize a holistic degree of stress suffered by aquatic plants (Bertrand et al. 2019). Endpoints during germination and root development are simple but sensitive, which are generally employed for assessment of phytotoxicity of chemicals to plants (Liu et al. 2017). Thus, effective concentrations that are obtained from quantitative structure-activity relationship model are widely used for ecological risk assessment of pollutants (Pan and Chu 2016). Therefore, in the present study, we investigate effects of ciprofloxacin on turions germination, root elongation, seedling development, and biomass accumulation, photosynthetic pigment contents, H_2O_2 content, lipid peroxidation degree, seven antioxidants of *P. crispus* and *H. verticillata*. The relationships between seedling tolerance and drug concentrations were established and inhibitory concentrations were obtained for evaluation of ciprofloxacin phytotoxicity to plants. Furthermore, physiological endpoints were integrated to reflect the holistic levels of stress on plants. Our aims were to (1) evaluate the sensitivity of different endpoints during germination and seedling phases to ciprofloxacin, (2) try to compare the tolerance of *P. crispus* and *H. verticillata* seedlings to ciprofloxacin, (3) to explore toxicity threshold of ciprofloxacin based on seedling tolerance, (4) to explore the stress levels of ciprofloxacin on both plants and reveal the physiological mechanism that was involved in plant adaptation to ciprofloxacin. This study might help to evaluate the ecotoxicity of ciprofloxacin to submerged species, predict their population dynamics under ciprofloxacin pollution and provide theoretical guidance for restoration of degraded shallow waters polluted by ciprofloxacin.

2. Materials And Methods

2.1 Plant materials and experimental design

P. crispus turions (green turions, weighing 0.68 ± 0.12 g, $n = 250$) were hand collected in September 2020 from Lake Diaocha ($30^{\circ}69'N$; $113^{\circ}72'E$) in Hubei Province, PR China. After cleaning thoroughly, the turions were transported into a climate chamber on 20 September. Turions of *H. verticillata* (weighing 0.15 ± 0.01 g, $n = 250$) were hand collected in January 2021 from Lake Diaocha. After cleaning thoroughly, the turions were transported into a climate chamber on 5 January.

During the whole experiment, the turions/seedlings of *P. crispus* or *H. verticillata* were cultivated in 10% Hoagland's solution in a transparent container (Hoagland and Arnon 1950) at $15 \pm 2^{\circ}C$ or $25 \pm 2^{\circ}C$ with a 12/12 light/dark cycle and a photon flux density of $30-40 \mu mol \text{ photons } m^{-2} s^{-1}$. The turions/seedlings were exposed to various concentrations of ciprofloxacin (0, 0.1, 1, 5 and 10 mg/L) and turions (seedlings) and nutrient solution without ciprofloxacin was set as the control group. The whole observation lasted for 35 days (d), where turions were kept in a solution volume of 2 L during germination (0-7d), and seedlings (8-35d) were kept in 5 L solution to meet enough space for their growth. Germination experiment was performed in five replicates with each replicate containing 10 uniform turions. At 8d, uniform seedlings from different treatments were respectively selected for further investigation, where each treatment had five replicates and each replicate contained five seedlings. To maintain approximately constant concentrations of ciprofloxacin and nutrients, they were changed every 48 hours during the whole observation. Germination

parameters were investigated in the first 7d, and rooting parameters were investigated at 6-14d. Seedling growth and tolerance indicators of seedlings were investigated at 7, 14, 21, 28 and 35d respectively. Plant biomass and physiological responses of seedlings were analyzed at 35d.

Ciprofloxacin used in the experiments was ciprofloxacin monohydrochloride monohydrate (Purity $\geq 98\%$; CAS NO. 86393-32-0), and was purchased from Aladdin Reagent Co., Ltd. (Shanghai, China).

2. 2 Ciprofloxacin concentrations analysis

The actual concentrations of ciprofloxacin were determined 1h after being added into the experimental containers, which were 0.1135 ± 0.0022 , 1.0483 ± 0.0047 , 4.5034 ± 0.0484 and 9.7506 ± 0.0868 mg/L ($n = 3$) for the nominal concentrations of 0.1, 1, 5 and 10 mg/L, respectively. Ciprofloxacin concentrations were analyzed by high performance liquid chromatography (HPLC, Agilent1100, USA). The mobile phase was 0.025 mol/L phosphoric acid (pH = 2.5, regulated by triethylamine) and acetonitrile, where the volume ratio of phosphoric acid and acetonitrile was 83:17, using C18 column (Agilent, 20RBAX SB-C18, 4.6 mm×250 mm, 5 μ m particle size), with a flow rate of 1.0 ml/min and excitation/emission wavelength at 278/453 nm. The limit of detection was 0.1 μ g/L. External standards were used for quantitative determination. The calibration curves showed good linearity for the analyte, with correlation coefficients of 0.9994.

2. 3 Estimation of germination and rooting

P. crispus turions were considered germinated when 5 mm of shoot had emerged, and the turions of *H. verticillata* were considered germinated once evident elongation (≥ 1 cm) of the buds (internodes) had emerged. Germination rate (percentage) and germination time were documented in the first 7d. For both species, rooting rate (percentage) and rooting time were recorded when 5 mm of radicle (root) had emerged. Both germination rate and rooting rate were displayed as x percentage (%) of the control. Germination time and rooting time were calculated according to Ellis and Roberts (1981):

$$\text{Germination time (days)} = \frac{\sum Dn}{\sum n} ,$$

$$\text{rooting time (days)} = \frac{\sum Dn}{\sum n} .$$

Where D was the number of days since the beginning of the experiment, n was the number of germinated/rooted turions on day D.

2. 4 Seedling growth, tolerance indicators and IC50 assessment

Shoot number and root number were used to assess growth status of both *P. crispus* and *H. verticillata* seedlings under ciprofloxacin treatment. Due to the structural differences between these two species, the shoot of *H. verticillata* referred to the stolon branch in this study. Root tolerance index (RTI) and shoot tolerance index (STI) were used to assess seedling tolerance to ciprofloxacin exposure. RTI and STI were calculated according to Rossato et al (2011):

$$\text{RTI (\%)} = \frac{\text{Length of the longest root in ciprofloxacin treatment}}{\text{Length of the longest root in the control}} \times 100,$$

$$\text{STI (\%)} = \frac{\text{Length of the longest shoot in ciprofloxacin treatment}}{\text{Length of the longest shoot in the control}} \times 100.$$

Inhibition rate (IR, %) based on RTI and STI was calculated according to the following equation:

$$\text{IR} = (\mu_0 - \mu_t) / \mu_0 \times 100,$$

Where μ_0 was the value of the control and μ_t was the corresponding value of treatment groups at the same sampling date. To acquire ciprofloxacin effect on seedling tolerance as time elapsed, regression models between the IR values and ciprofloxacin concentrations were established at 7, 14, 21, 28 and 35d using linear regression techniques performed in R software (version 4.0.4), and the inhibitory concentrations (IC₅₀, inhibitory concentration causing 50% of an endpoint) with a 95% confidence interval (95% CI) were calculated. Regression models and details were shown in **supplementary information**.

2. 5 Plant biomass

At 35d, after blotting water with filter paper, the shoot biomass and root biomass of both *P. crispus* and *H. verticillata* seedlings were estimated by an electronic analytical balance (accuracy 0.1 mg), where the biomasses were fresh weights (FW).

2. 6 Photosynthetic pigment measurement

The contents (mg/g FW) of photosynthetic pigment were determined based on the method of Jampeetong and Brix (2009) with some modifications. Fresh plant leaves (0.1g, FW) were cut into pieces and placed in 5-ml flasks. Then, 5 ml 95% ethanol was added into the flasks to extract the photosynthetic pigment in the dark for 24 h. Plant pigment contents were determined with a spectrophotometer at 470, 649 and 645 nm for chlorophyll a, chlorophyll b and carotenoids, respectively. Values were calculated according to Lichtenthaler and Wellburn (1983). All spectrophotometric analyses involved in this experiment were accomplished with a MAPADA UV-1200 spectrophotometer (Shanghai Meipuda Instrument Co. Ltd., Shanghai, China).

2. 7 Lipid peroxidation measurement

Malondialdehyde (MDA) content (μmol/g FW) was used as a proxy of lipid peroxidation level. Plant leaves (0.1g, FW) were homogenized using 3 ml 10% (w/v) trichloroacetic acid (TCA), and then the homogenate was centrifugated at 4000 rpm for 10 min at 4°C. For every 1mL of supernatant, 1 mL 0.6% (w/v) thiobarbituric acid (TBA) was added. The reaction mixture was heated at 95°C for 25 min and centrifugated at 4000 rpm for 10 min at 4°C after being cooled quickly. The resulting supernatant was analyzed at 450, 532 and 600 nm, respectively (Cang and Zhao 2013).

2. 8 Measurements of enzyme activity, soluble protein and H₂O₂ content

Plant leaves (0.1g, FW) were homogenized using 50 mM phosphate buffer solution (pH 7.8) containing 1% (w/v) polyvinyl pyrrolidone (PVPP) and 0.1 mM ethylenediaminetetraacetic acid (EDTA) at 4°C. Then, the homogenate was centrifuged at 8000×g for 15 min at 4°C. The resulting supernatant was used for

superoxide dismutase (SOD), catalase (CAT), guaiacol peroxidase (POD), polyphenol oxidase (PPO) and ascorbate peroxidase (APX) activity, soluble protein and hydrogen peroxide (H_2O_2) content assays.

SOD activity was measured at 560 nm according to Cang and Zhao (2013). The reaction mixture was 50 mM phosphate buffer solution (pH 7.8) containing 13 mM methionine, 0.075 mM NBT (nitro blue tetrazolium), 0.1 mM EDTA (ethylene diamine tetraacetic acid), 0.002 mM riboflavin and enzyme extract. The unit (U) of SOD activity (U/mg protein) was defined as the amount that resulted in a 50% inhibition of the initial rate of the reaction in the absence of the enzyme.

CAT activity was determined spectrophotometrically at 240 nm based on the method of Cang and Zhao (2013). The reaction mixture contained 200 mM phosphate buffer solution (pH 7.8), 100 mM H_2O_2 and enzyme extract. The unit of CAT activity (U/min • mg protein) was defined as a decrease of 0.1 in absorbance at 240 nm in 1 min.

POD activity was measured based on the guaiacol method (Zhang et al. 2009), and the reaction mixture comprised 100 mM potassium phosphate buffer, 20 mM guaiacol, 65mM H_2O_2 and enzyme extract. One unit of POD activity (U/min • mg protein) was defined as an absorbance change of 0.01 at 470 nm in 1 min.

PPO activity was determined according to Shi (2016), the reaction mixture was 50 mM phosphate buffer solution (pH 6.4) containing 100 μM catechol and enzyme extract, with a per-minute absorbance change of 0.01 at 398nm as one unit of PPO activity (U/min • mg protein).

APX activity was measured using the method of Chen et al. (2017), and the reaction solution was 50 mM phosphate buffer (pH 7.8) containing 2 mM EDTA, 5 mM AsA (ascorbic acid), 20 mM H_2O_2 and enzyme extract. One unit of APX activity (U/min • mg protein) corresponded to a per-minute absorbance change of 0.01 at 290 nm.

H_2O_2 content ($\mu\text{mol/g}$ FW) was determined according to Shi (2016). Briefly, for every 1 mL enzyme extract, 1 mL 5% (w/v) titanium sulfate was added. After 10 min, the reaction solution was centrifuged at 12000 rpm for 10 min at 4°C and the supernatant was then analyzed at 410 nm.

Soluble protein content was determined according to Bradford (1976) and the bovine serum albumin was used as standard. Briefly, the reaction mixture contained 5 mL Coomassie brilliant blue G-250 solution and 0.1 mL crude extract. After 2 min of reaction, the soluble protein content (mg/g FW) was calculated by recording the absorbance at 595 nm.

2. 9 Measurement of non-enzymatic antioxidants

Reduced ascorbic acid (AsA) was determined according to Cang and Zhao (2013) with some modifications. Plant leaves (0.1g, FW) were ground by 4 mL 5% (w/v) TCA and then centrifuged at 4000 rpm for 10 min at 4°C. Then 1 mL supernatant was mixed with 1 mL 5% (w/v) TCA, 1 mL absolute ethanol, 0.5 mL 0.4% (w/v) H_3PO_4 in absolute ethanol, 1 mL 0.5% (w/v) red phenanthroline (BP) in absolute ethanol and 0.5 mL 0.03%

(w/v) FeCl₃. The reaction mixture was then incubated at 30°C for 40 min, and the absorbance at 534 nm was recorded. And a standard curve with AsA was used.

Reduced glutathione (GSH) was determined according to Cang and Zhao (2013) with some modifications. Plant leaves (0.1 g, FW) were ground by 3 mL 5% (w/v) TCA and then centrifuged at 12000 rpm for 20 min at 4°C. The reaction mixture contained 1 mL supernatant, 1 mL 100 mM phosphate buffer (pH 7.7) and 0.5 mL 4 mM DTNB (5,5'-dithio (2,2'-dinitro) benzoate) in 100 mM phosphate buffer (pH 6.8). After being incubated at 25°C for 10 min, the absorbance of the reaction mixture was recorded at 412 nm.

2. 10 Integrated biomarker response

To visualize and compare the stress degree among different treatment groups, the integrated biomarker response (IBR) including MDA, H₂O₂, SOD, CAT, POD, PPO, APX, AsA and GSH were computed according to Kim et al. (2010). The detailed analysis process was shown in **supplementary information**. Spearman correlation coefficients were calculated between the IBR levels in both species and ciprofloxacin concentrations.

2. 11 Statistical analyses

All values were expressed as the mean ± standard deviation (SD). The Levene and Kolmogorov-Smirnov tests were used to verify homoscedasticity and normality criteria, respectively. One-way analysis of variance (ANOVA) was used for the statistical analysis. And a least significant difference (LSD) test was used to separate differences between pairs of treatments, where all the differences were considered significant at $p < 0.05$ (SPSS 23.0, IBM Inc., Chicago, IL, USA). Graphs were produced using Sigma-Plot 12.5 (Systat Software, Inc., USA). No shoots and roots of *H. verticillata* formed at 7d and thus no relevant analysis was performed then.

3. Results

3. 1 Germination and rooting

Germination rates of both *P. crispus* and *H. verticillata* were not affected by ciprofloxacin exposure ($p > 0.05$; Fig. 1A). Rooting rate of *P. crispus* decreased slightly but not significantly with rising ciprofloxacin concentrations ($p > 0.05$; Fig. 1B) while it showed no significant changes in *H. verticillata* ($p > 0.05$; Fig. 1B)

Ciprofloxacin did not affect the germination time of *P. crispus* ($p > 0.05$; Fig.1C), while 0.1 mg/L treatment significantly decreased the germination time of *H. verticillata* ($p < 0.05$; Fig.1C). 10 mg/L ciprofloxacin significantly increased the rooting time of *P. crispus* (by 42.5%) when compared with the control ($p < 0.05$; Fig.1D). Rooting time of *H. verticillata* also showed slight increases with increasing ciprofloxacin concentrations, reaching levels significantly higher than the control in 5 mg/L treatment ($p < 0.05$; Fig.1D).

3. 2 Tolerance indicators and seedling growth

When compared with the control, ciprofloxacin at 1–10 mg/L and 0.1–10 mg/L significantly decreased RTI of *P. crispus* at 7-21d and 28-35d, respectively ($p < 0.05$; Fig. 2A). Moreover, RTI of *P. crispus* treated with 1–5 mg/L decreased significantly over time. *H. verticillata* treated with ciprofloxacin concentrations of 1–10 mg/L and 0.1–10 mg/L showed significantly lower RTI compared to the controls at 14d and 21-35d, respectively ($p < 0.05$; Fig. 2B). Regardless of ciprofloxacin concentrations, RTI of *H. verticillata* decreased significantly over time ($p < 0.05$).

The STI of *P. crispus* was not affected by ciprofloxacin at 7d ($p > 0.05$; Fig. 2C), but decreased significantly at ciprofloxacin concentrations of 5–10 mg/L and 1–10 mg/L after 14d and 21-35d, respectively ($p < 0.05$). At 14d, ciprofloxacin at all concentrations slightly increased the STI of *H. verticillata* ($p > 0.05$; Fig. 2D). At 21d, the STI of *H. verticillata* increased significantly at 0.1 mg/L ciprofloxacin but decreased significantly at 10 mg/L ($p < 0.05$). At 28 and 35d, it was significantly lowered by ciprofloxacin at 0.1–10 mg/L and 1–10 mg/L, respectively ($p < 0.05$). Additionally, STI of both *P. crispus* and *H. verticillata* treated with 1–5 mg/L ciprofloxacin decreased significantly over time ($p < 0.05$).

When compared to the controls, root number of *P. crispus* showed no significance at 7-14d but significant decreases in 1–10 mg/L treatments at 21-35d ($p < 0.05$, Fig. 2E). Additionally, root number of *P. crispus* under 1 mg/L ciprofloxacin showed significant increases over time ($p < 0.05$), while plants treated with 5 and 10 mg/L showed no changes ($p > 0.05$) from 21d to 35d, and from 7d to 35d, respectively. At 14-21d and 28-35d, root number of *H. verticillata* decreased significantly with ciprofloxacin concentrations of 1–10 mg/L and 0.1–10 mg/L, respectively ($p < 0.05$; Fig. 2F). Under 0.1 mg/L ciprofloxacin, root number of *H. verticillata* increased significantly over time ($p < 0.05$), without such increases observed at higher ciprofloxacin concentrations during 21-35d ($p > 0.05$). These results indicated inhibition effect of ciprofloxacin at higher concentrations on the root development of both *P. crispus* and *H. verticillata* seedlings especially at later stages of exposure.

Regardless of time points, shoot number of *P. crispus* decreased slightly with rising ciprofloxacin concentrations while *H. verticillata* showed no alterations compared with their corresponding controls ($p > 0.05$; Fig. 2G-H). Additionally, with time prolonging, shoot number of *P. crispus* did not change significantly ($p > 0.05$), while it increased significantly in *H. verticillata* regardless of ciprofloxacin concentrations ($p < 0.05$).

3. 3 Time-dependent IC50 based on STI and RTI

IC50 values of ciprofloxacin based on RTI and STI of both species decreased over time (Table 1), indicating tolerance of these two plant species was attenuated with prolonged exposure of ciprofloxacin. What's more, regardless of time and species, IC50 values based on RTI were respectively lower than those based on STI, suggesting RTI was more sensitive to ciprofloxacin exposure.

Table 1
Time-dependent IC50 values of ciprofloxacin based RTI and STI (mg/L, 95% CI).

Time (day)	<i>P. crispus</i>	<i>H. verticillata</i>		
	RTI	STI	RTI	STI
7	3.405 (2.458, 4.352)	NA ^a	NA	NA
14	3.308 (2.260, 4.356)	5.566 (4.143, 6.989)	5.896 (4.388, 7.404)	NA
21	3.133 (2.392, 3.874)	4.487 (3.661, 5.313)	3.492 (2.811, 5.074)	10.227 (6.661, 13.792)
28	2.302 (1.246, 3.357)	3.876 (3.016, 4.735)	2.939 (1.970, 3.908)	6.367 (4.930, 7.803)
35	2.203 (1.139, 3.267)	3.453 (2.582, 4.324)	2.635 (1.336, 3.934)	5.636 (4.405, 6.867)

NA^a referred to “not available” due to lack of significance of the models (no calculation of the IC50 values), or because no radicle or shoot emerged then.

3. 4 Plant biomass

As shown in Fig.3A, B, ciprofloxacin at 1–10 mg/L significantly decreased both the shoot biomass and root biomass of *P. crispus* ($p<0.05$). Significant decreases of the biomasses in *H. verticillata* were observed at ciprofloxacin concentrations of 0.1–10 mg/L ($p<0.05$).

3. 5 Photosynthetic pigment contents

For *P. crispus*, when compared with the corresponding controls, ciprofloxacin at concentrations of 0.1–10 mg/L, 0.1–10 mg/L and 5–10 mg/L significantly decreased the contents of chlorophyll a (by 16.53%-92.71%), chlorophyll b (by 19.71%-92.84%) and carotenoid (by 71.24%-78.29%), respectively ($p<0.05$; Fig.4A). Contents of chlorophyll a, chlorophyll b and carotenoid of *H. verticillata* decreased significantly by 58.97%-88.54%, 51.63%-83.91% and 71.24%-78.29% with ciprofloxacin concentrations of 1–10 mg/L when compared with their controls ($p<0.05$; Fig.4B).

3. 6 Physiological responses

Ciprofloxacin had no significant effects on MDA content of both *P. crispus* and *H. verticillata* (Fig. 5, 6A; $p>0.05$). H₂O₂ content of *P. crispus* and *H. verticillata* was significantly elevated (by 16.02%-27.08% and 29.82%-42.06%, respectively) by ciprofloxacin concentrations of 1–10 mg/L compared to the controls (Fig. 5, 6B; $p<0.05$).

As shown in Fig.5C, ciprofloxacin sharply elevated SOD activity of *P. crispus* ($p<0.05$), reaching levels 1.6-5.0 times higher than the control. However, SOD activity of *H. verticillata* showed no significant differences when compared with the control (Fig.6C; $p>0.05$). CAT activity of *P. crispus* was significantly inhibited, which declined by up to 22.9%, 40.4% and 59.1% at 1, 5 and 10 mg/L ciprofloxacin compared with the control, respectively (Fig.5D; $p<0.05$). Similarly, CAT activity of *H. verticillata* was declined by up to 17.2%, 45.1% and 36.6% at 1, 5 and 10 mg/L ciprofloxacin compared with the control, respectively (Fig.6D; $p<0.05$). No significance was found in POD activity of *P. crispus* at lower concentrations of ciprofloxacin (0.1-1.0 mg/L; $p>0.05$), while it was 17.8–26.1 times higher than the control at 5–10 mg/L ciprofloxacin (Fig.5E; $p<0.05$). POD activity of *H. verticillata* showed no changes in 0.1 mg treatment but was increased by up to 66.7%-87.6% in 1–10 mg/L treatments in relative to the control (Fig.6E; $p<0.05$). PPO activity of *P. crispus* was significantly inhibited by ciprofloxacin (Fig.5F; $p<0.05$), representing decreases of 40.3%-52.3% compared to the control. However, PPO activity of *H. verticillata* was significantly induced by ciprofloxacin (Fig.6F; $p<0.05$), representing increases of 28.4%-53.1% compared to the control. APX activity of *P. crispus* was significantly induced by ciprofloxacin concentrations of 1–5 mg/L (Fig.5G; $p<0.05$), without any significant differences in other treatments. In *H. verticillata*, APX activity represented an increase of 101.7% at 10 mg/L ciprofloxacin compared to the control (Fig.6G; $p<0.05$), without significant increases at lower ciprofloxacin concentrations.

AsA content of *P. crispus* was significantly elevated by ciprofloxacin, reaching levels 0.3–4.9 times higher than the control (Fig.5H; $p<0.05$). AsA content of *H. verticillata* displayed an increasing tendency with increasing ciprofloxacin concentrations, reaching levels 1.3, 2.0 and 3.4 times higher than the control at 1, 5 and 10 mg/L ciprofloxacin, respectively (Fig.6H; $p<0.05$). When compared to the control, no difference was found in GSH content of *P. crispus* at lower ciprofloxacin concentrations (0.1-1.0 mg/L; $p>0.05$), while it was increased by up to 96.4%-414.6% at 5–10 mg/L ciprofloxacin (Fig.5I; $p<0.05$). Similarly, GSH content of *H. verticillata* showed no significant changes at ciprofloxacin concentrations of 0.1-5 mg/L (Fig.6I; $p>0.05$), while it was elevated by 94.2% at the highest ciprofloxacin concentration ($p<0.05$).

3. 7 Integration of biomarker responses

The scores and IBR values were computed and displayed in Table 2. With rising ciprofloxacin concentrations, the IBR values of both species tended to increase. Additionally, the IBR values of both *P. crispus* and *H. verticillata* showed good positive correlations with ciprofloxacin concentrations ($r= 0.991$, $p< 0.05$; $r= 0.943$, $p< 0.05$, respectively), indicating that higher concentrations of ciprofloxacin caused higher degree of stress on these two species.

Table 2

The scores of biomarkers and integrated biomarker response (IBR) values in *P. crispus* and *H. verticillata* exposed to different ciprofloxacin concentrations.

Biomarkers	<i>P. crispus</i>					<i>H. verticillata</i>				
	Control	0.1 mg/L	1 mg/L	5 mg/L	10 mg/L	Control	0.1 mg/L	1 mg/L	5 mg/L	10 mg/L
MDA	1.06	2.25	0.63	0.00	0.25	0.27	0.45	1.27	0.09	0.00
H ₂ O ₂	0.00	0.00	1.80	1.27	1.67	0.00	0.69	1.58	2.16	2.21
SOD	0.00	1.43	0.92	1.63	3.04	1.06	0.00	0.84	1.21	1.74
CAT	0.58	0.00	1.54	2.16	3.00	0.00	0.09	0.81	2.14	1.76
POD	0.00	0.06	0.14	2.07	3.05	0.00	0.09	1.56	1.98	2.05
PPO	0.00	1.80	1.80	1.56	2.04	0.00	1.46	2.21	0.50	1.25
APX	0.00	0.52	0.66	3.07	0.46	0.12	0.00	0.34	0.75	2.18
AsA	0.00	0.13	0.51	1.93	2.45	0.00	0.25	1.03	1.54	2.64
GSH	0.16	0.00	0.03	0.82	2.97	0.15	0.21	0.37	0.00	1.56
IBR value	0.06	0.36	2.00	8.23	12.86	0.01	0.19	3.44	3.91	8.24

4. Discussion

Many studies reported that seed germination of crops was insensitive to various antibiotics such as fluoroquinolones, lincosamides, macrolides, sulfonamides, tetracyclines (Hillis et al. 2011; Bellino et al. 2018; Pan and Chu 2016). In our study, ciprofloxacin employed did not influence both the germination rate and rooting rate of *P. crispus* and *H. verticillata*, which suggested that the effects of antibiotics on turion germination were nearly not available. Some cellular processes might be negatively affected by antibiotics (Gambonnet et al. 2001), but germination was considered as a highly conserved process during which sufficient nutrients, carbohydrates, and proteins were stored and available for the appearance of germ or radicle (Hillis et al. 2011). Turions functionally resemble seeds, serving multiple functions including carbohydrate storage, propagation, and dispersal (Jian et al. 2003). In this light, germination and rooting of turions of *P. crispus* and *H. verticillata* could be considered tolerant to ciprofloxacin exposure. Notably, without impacts on germination and rooting rates, ciprofloxacin could accelerate or delay the germination and rooting process, which were assessed by germination time and rooting time. Indeed, we found ciprofloxacin at 0.1 mg/L significantly accelerated turion germination of *H. verticillata*, with slight accelerations at higher concentrations. Similar results have also been found in maize seeds, where ciprofloxacin was proved to induced H₂O₂ production by inhibiting the activity of mitochondrial Complex III (Gomes et al. 2019). Consequently, elevated H₂O₂ promoted the signaling for germination and accelerate seed germination of maize (Gomes et al. 2019; Wojtyla et al. 2016). However, though phytochrome-induced transmissible signals, low molecular mass carbohydrates and phytohormones were proposed to be the

possible signals regulating turion germination (Tirlapur et al. 1999; Ley et al. 1997), no specific metabolic changes during the breaking period could be served as metabolic signals (Adamec, 2018). Taken ciprofloxacin as an inhibitor of mitochondrial Complex III (Gomes et al. 2019), further studies on energy metabolism, together with saccharides contents might help to explain beneficial effects of ciprofloxacin on turion germination. Rooting, as the first step of development of the root system, plays an essential role for plant life. Initiation of roots of higher plants were mediated by phytohormones and many external factors such as light, temperature, nutrients and cutting (Xuan et al. 2012). For example, as an important signal for cell division, elongation and differentiation, auxin was highly involved in root primordia formation (Pagnussat et al. 2004). Additionally, nitric oxide was a main factor that mediated the auxin response during rooting process (Pagnussat et al. 2004). Ciprofloxacin has been proved to disturb cellular nitric oxide production (Aiassa et al. 2006; Kolios et al. 2006; Adedara et al. 2021), resulting in lipid, protein, and nucleic acids modification and cellular chaos. Our results showed that ciprofloxacin especially at higher concentrations (5–10 mg/L) had retarding effects on the rooting process of *H. verticillata* and *P. crispus*, and these might because ciprofloxacin disturbed the cellular nitric oxide level, which could further impact auxin response as well as rooting performance of turions.

Regardless of exposure time, RTI of both species decreased significantly with increasing ciprofloxacin concentrations, indicating toxicity of ciprofloxacin on RTI was concentration-dependent. Unlike RTI, STI of *P. crispus* at 7d was not influenced by ciprofloxacin treatment. What's more, ciprofloxacin promoted STI of *H. verticillata* at 14-21d. This might because ciprofloxacin addition provided more carbon and nitrogen sources for plant growth (Mao et al. 2021), resulting in but better growth performance and higher STI over these short exposures. However, after longer exposures, STI of both species also decreased with increasing ciprofloxacin concentrations, indicating hysteresis effects of ciprofloxacin toxicity on STI. RTI of *P. crispus* treated with 1–5 mg/L ciprofloxacin and *H. verticillata* treated with 0.1-5 mg/L ciprofloxacin decreased significantly over exposure time, with STI of both species in 1–5 mg/L treatments decreasing significantly with time prolonging. These results suggested seedling tolerance of *H. verticillata* and *P. crispus* to ciprofloxacin decreased over time, and both RTI and STI were reliable and sensitive indicators for ciprofloxacin toxicity on plant early establishment. Moreover, considering that IC50 values for RTI were lower than those for STI, we concluded that RTI was more sensitive than STI, regardless of species and exposure time. Previous studies also demonstrated high sensitivity of root elongation to antibiotics including tetracycline, sulfamethazine, norfloxacin, erythromycin and chloramphenicol compared to shoot elongation (Pan and Chu 2016). Root number and shoot number could reflect seedling growth status directly. Regardless of exposure time, root number of both species decreased significantly with increasing ciprofloxacin concentrations, while shoot number was less affected. Roots are the main organ for absorbing antibiotics and growth of roots was more severely inhibited than that of shoots (Guo et al. 2020), presumably because seedlings of *H. verticillata* and *P. crispus* mainly relied on their roots to absorb ciprofloxacin. Accordingly, reductions in root number and root biomass might help to avoid the absorption of larger quantities of toxicants and thus reducing damages to seedling growth. Regardless of ciprofloxacin concentration, shoot number of *H. verticillata* increased over time, while *P. crispus* showed no such growth in its shoot. These results indicated better adaptability of *H. verticillata* than *P. crispus* under ciprofloxacin exposure.

With RTI, STI and root number decreasing with rising ciprofloxacin concentrations and time prolonging, shoot and root biomass accumulations of both *H. verticillata* and *P. crispus* were evidently inhibited. On one hand, this may be explained by toxicity effect of ciprofloxacin on photosynthetic pigments. Fluoroquinolone antibiotics were reported to disrupt chloroplast structure and eventually resulted in reductions of pigment contents as well as etiolation of plant leaves (Liu et al. 2021; Yan et al. 2019). Furthermore, damaged leave functional traits would inevitably impact plant photosynthesis and subsequent growth processes. On the other hand, plants tend to defense against stress but actively inhibit growth related metabolisms to respond to adverse environmental conditions (Zhang et al. 2020). And worse growth performance of *P. crispus* and *H. verticillata* might be related to plant defense against oxidative stress induced by ciprofloxacin, during which cellular energy was highly consumed and thus energy available for growth process might be limited.

Ciprofloxacin could cause oxidative stress by inhibiting mitochondria electron transport chain, leading to cellular ROS accumulation and oxidative damages (Gomes et al. 2019). For example, ROS could attack and degrade the cell membrane to produce MDA, which functioned as a proxy of lipid peroxidation. No lipid peroxidation was observed in both species under ciprofloxacin exposure. However, H_2O_2 contents of *P. crispus* and *H. verticillata* were elevated by 16.02%-27.08% and by 29.82%-42.06%, respectively. Antibiotics like fluoroquinolones could induce hormesis in various plant species and cause promotions of several physiological, cellular and growth traits of plants, where the observed stimulatory responses were generally lower than 1.5 times of the control (Agathokleous et al. 2018). Thus, the H_2O_2 levels in *P. crispus* and *H. verticillata* were probably below those at which H_2O_2 would become phytotoxic. Though with oxidative cellular toxicity, H_2O_2 is an important signal molecule in modulating signaling networks that regulates plant growth, development and stress responses (Mittler et al. 2004).

To cope with oxidative event, plants are capable of activating their ROS detoxification systems, the enzymatic and non-enzymatic antioxidants. Firstly, superoxide anions were transferred into H_2O_2 by SOD, and then H_2O_2 could be further transferred into O_2 by CAT, POD and APX (Yan et al. 2019). PPO could protect plant from photo-oxidative damages by catalyzing the oxidation of phenols (Boeckx et al. 2015). Both AsA and GSH are powerful antioxidant compounds in scavenging cellular ROS directly, by which plants could enhance their antioxidant capacity (Mittler et al. 2004). In *P. crispus*, activated SOD activity produced large quantities of H_2O_2 . Meanwhile, POD and APX activities, AsA and GSH contents were significantly elevated. These antioxidants worked well in removing ROS induced by ciprofloxacin, thereby controlling H_2O_2 levels tightly and maintaining stability of cell membranes in *P. crispus*. However, CAT and PPO activities responded negatively under ciprofloxacin exposure. As a photosensitizer, CAT would be likely inactive once photosynthetic pigment contents were reduced (Smirnoff 1995), which was in line with the observed reductions in pigments contents. Decreased activity of PPO has been considered as one of the outcomes of plant enhanced antioxidant capacity (Boeckx et al. 2015), indicating the local oxygen was maintained at levels that could not cause photoinhibition or oxidative damages. For *H. verticillata*, SOD activity increased slightly but not significantly in 5–10 mg/L treatments. At the same time, activated POD and APX could remove H_2O_2 catalyzed by SOD activity. Compared with *P. crispus*, the effects of SOD in detoxifying ROS in *H. verticillata* was relatively weaker. This might because the quantities of superoxide anions were beyond the capacity of SOD, which would inhibit the outputs of this enzyme. Likely, as a

compensative response, PPO activity was significantly activated, indicating high amounts of oxygen were removed by the oxidation of phenolic compounds. Additionally, AsA and GSH contents increased markedly especially at high ciprofloxacin concentrations, indicating their vital roles in quenching ROS and protecting cells from oxidative damages. To conclude, firstly, though *P. crispus* and *H. verticillata* employed different antioxidant enzymes to prevent oxidative damages, AsA and GSH worked well in ROS detoxification in both species. Both AsA and GSH are water soluble and easily accessible with potent antioxidant properties. Furthermore, addition of exogenous antioxidants could increase their endogenous contents and enhance plant antioxidant capacity under stress conditions (Akram et al. 2017). Therefore, our results might provide some research ideas for alleviation of phytotoxicity of ciprofloxacin. Secondly, the POD-H₂O₂ decomposition system was involved in the degradation of chlorophylls (Kar and Choudhuri 1987), and the observed elevations of POD activity could be invoked to account for the reductions of chlorophylls contents in both species. Thirdly, under exposure to antibiotics like ciprofloxacin, defense against oxidative stress could elevate cellular energy consumption, causing lower amounts of energy available for growth (Aderemi et al. 2018). To speculate, *P. crispus* and *H. verticillata* might allocate much more cellular energy to detoxify ROS to maintain physiological stability, resulting in growth inhibitions related to insufficient energy supply.

IBR levels were employed to compare stress degree related to various environmental conditions, where a higher IBR level indicated more serious impact in an organism (Kim et al. 2010). We found IBR values increased with rising ciprofloxacin concentrations. That was, the higher the ciprofloxacin concentrations were, the more serious the damage to both submerged plants. These results were consistent with those of seedling tolerance, photosynthetic pigment contents and biomass accumulations, suggesting toxicity effects of ciprofloxacin on these two species were concentration-dependent. On the other hand, the selected biomarkers responded efficiently to ciprofloxacin pollution, and the IBR could serve as a reliable parameter for quantitative evaluation of the toxicological effects of ciprofloxacin toward aquatic plants.

5. Conclusion

Germination rate and rooting rate of turions of *P. crispus* and *H. verticillata* were not impacted by ciprofloxacin employed in this study. However, ciprofloxacin delayed rooting process of both species. Both RTI and STI were reliable and sensitive endpoints to assess ciprofloxacin toxicity on seedling development. Additionally, roots came to be more sensitive than shoots under ciprofloxacin treatment. The lowest toxicity threshold (IC₅₀) values of ciprofloxacin based on seedling tolerance of *P. crispus* and *H. verticillata* were 2.203 mg/L and 2.635 mg/L, respectively. With better growth performance of shoots, *H. verticillata* appeared to be more tolerant to ciprofloxacin exposure than *P. crispus*, which might provide information for the selection of aquatic plant species for restoration of water bodies polluted by ciprofloxacin. Ciprofloxacin induced oxidative stress and activated the antioxidant system of both species. Key antioxidant enzymes for ROS detoxication differed between *P. crispus* and *H. verticillata*, while non-enzymatic antioxidants (AsA and GSH) functioned well in both species, which might provide valuable research ideas for alleviation of ciprofloxacin toxicity. As the first study that investigated ciprofloxacin toxicity on germination and seedling development of submerged turions, this research might provide a new perspective to reveal the aquatic environments risks of antibiotics pollution. Stress levels suffered by both plants were enhanced with rising

drug concentrations, suggesting IBR could serve as a reliable parameter for quantitative evaluation of the toxicological effects of ciprofloxacin toward aquatic plants. Lastly, to better predict population dynamics and improve effectiveness of vegetation restoration, the combined effects of antibiotics and other pollutants on propagule germination and early establishment of aquatic plants should be further studied.

Declarations

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Author contributions

Pei Fan performed the study, analyzed the data and wrote the paper. Chunhua Liu reviewed and edited the paper. Zhen Ke and Wei Zhou performed the sample collection and sample processing. Zhonghua Wu provided experimental supervision.

Data availability

All the Data for this study are available from Zhonghua Wu (wuzhonghua@whu.edu.cn) and Pei Fan (fanpei@whu.edu.cn).

Ethics declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

All authors declared that there were no competing interests.

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Figures

Figure 1

Germination rate (A), rooting rate (B), germination time (C) and rooting time (D) of *P. crispus* and *H. verticillata* under ciprofloxacin exposure. Bars represent the mean of five replicates $\pm$ standard deviation. Different letters indicate significant differences among different treatments for a specific plant species ($p < 0.05$, LSD test).

Figure 2

Root tolerance index (A, B), shoot tolerance index (C, D), root number (E, F) and shoot number (G, H) of *P. crispus* and *H. verticillata* after 7, 14, 21, 28 and 35d of ciprofloxacin exposure. Bars represent the mean of five replicates $\pm$ standard deviation. Stars indicate significant differences among different time points at the same ciprofloxacin concentration ($p < 0.05$, LSD test).

Figure 3

Biomass of *P. crispus* (A) and *H. verticillata* (B) after 35d of ciprofloxacin exposure. All values represent the mean of five replicates $\pm$ standard deviation. Different letters represent statistically significant differences among different treatments for a specific endpoint ($p < 0.05$, LSD test).

Figure 4

Pigment contents of *P. crispus* (A) and *H. verticillata* (B) after 35d of ciprofloxacin exposure. All values represent the mean of five replicates $\pm$ standard deviation. Different letters represent statistically significant differences among different treatments for a specific endpoint ($p < 0.05$, LSD test).

Figure 5

MDA content (A), H₂O₂ content (B), SOD (C), CAT (D), POD (E), PPO (F) and APX (G) activity, AsA content (H) and GSH content (I) of *P. crispus* exposed to ciprofloxacin. Bars represent the mean of five replicates $\pm$ standard deviation. Different letters represent statistically significant differences among different treatments ($p < 0.05$, LSD test).

Figure 6

MDA content (A), H₂O₂ content (B), SOD (C), CAT (D), POD (E), PPO (F) and APX (G) activity, AsA content (H) and GSH content (I) of *H. verticillata* exposed to ciprofloxacin. Bars represent the mean of five replicates $\pm$ standard deviation. Different letters represent statistically significant differences among different treatments ($p < 0.05$, LSD test).

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