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Inhibition of photosynthesis in cucumber leaves by oxybenzone

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Abstract

Oxybenzone (OBZ), a widely used sunscreen additive, has damaging effects on corals and plants. This study demonstrated that OBZ significantly inhibited photosynthesis and growth of cucumber (*Cucumis sativus* L.) seedlings. Combined analyses of chlorophyll fluorescence and 820-nm reflection suggested that OBZ severely inhibited photosynthetic electron transport (PET) from Q_A to cytochrome b_6f (Cyt b_6f) complex, restricting the reduction of the reaction center in PSI. Separate measurements of PSI and PSII PET in chloroplasts indicated that OBZ inhibited the PET of neither PSII nor PSI, but it severely inhibited the whole linear PET. We thus confirmed that the inhibition of PET by OBZ was due to the inhibition of plastoquinones (PQs) and/or the Cyt b_6f complex between PSI and PSII. PET is essential for producing ATP and NADPH used in CO_2 assimilation. Therefore, limiting the use of sunscreens containing OBZ is significant in protection of aquatic and terrestrial ecosystems and ensuring the safety of agricultural production.

Additional key words: carboxylation efficiency; fluorescence transient; gas exchange; JIP-test.

Introduction

Oxybenzone (OBZ), a popular additive in sunscreen, can protect human skin by blocking ultraviolet (UV) rays from the sun (Baker *et al*. 2015). In addition to being used in sunscreens, OBZ is also widely used in perfumes, shampoos, lip balms, and other pharmaceuticals and personal care products (PPCPs) (Aguirre *et al*. 1992, Emonet *et al*. 2001, Kasichayanula *et al*. 2007). OBZ can enter rivers, lakes, groundwater, sewage, and so on through human activities and was identified as an 'emerging environmental pollutant' by the Environmental Protection Agency of the USA (Rodil *et al*. 2012, Agüera *et al*. 2013). With the rise in coastal tourism, a large number of coral reefs in coastal areas are being exposed to water containing OBZ (Barón *et al*. 2013, Tashiro and Kameda 2013). OBZ, as a pseudo-persistent pollutant, has a long-term toxic effect on the environment (Vione *et al*. 2013).

Downs *et al*. (2016) observed that OBZ damaged symbiotic algae and lead to death of coral. This compound also causes photobleaching of corals and damage of aquatic ecosystem (Downs *et al*. 2016). Mao *et al*. (2017) showed that algae could absorb OBZ in culture solution, resulting in a decrease in chlorophyll (Chl) content and the inhibition of growth. Recently, the toxic effects of OBZ on organisms have attracted attention. Many coastal regions have begun to prohibit the use of sun creams that contain OBZ and other harmful chemicals (*e.g.*, Hawaii in the USA, marine ecoparks in Mexico, and the Pacific nation of Palau). The media has frequently called on swimmers to reduce the use of body creams containing OBZ. However, many skin experts as well as many sun cream manufactures claim that this policy lacks supporting academic evidence from third parties. Other factors may lead to coral poisoning. To date, most research on OBZ has mainly emphasized animals and humans (Aguirre *et al*. 1992, Lenique *et al*. 1992, Emonet *et al*. 2001, Kasichayanula *et al*. 2007), whereas a few studies on plants have mainly focused on the absorption of OBZ, transformation of OBZ, and resulting changes in the antioxidant system (Chen *et al*. 2017, 2018).

Photosynthesis is one of the most important pathways in plants. More than 90% of a plant's dry matter is derived from photosynthesis. If OBZ can inhibit plant photosynthesis, then almost all plants, including algae and lower and higher plants, can be damaged by OBZ. Our recent study showed that OBZ immediately inhibited photosynthetic electron transport (PET) in chloroplasts of cucumber plants, resulting in the overproduction of reactive oxygen species (ROS) (Zhong *et al*. 2019a).

However, the mechanisms of inhibiting photosynthesis by OBZ is still unknown. Furthermore, our previous study used detached leaves, and whether OBZ can damage higher

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Abbreviations: CE – carboxylation efficiency; Chl – chlorophyll; C_i – intercellular CO_2 concentration; Cyt b_6f – cytochrome b_6f; DCMU – 3-(3,4-dichlorophenyl)-1,1-dimethyl-urea; DCPIP – 2,6-dichlorophenolindophenol; g_s – stomatal conductance; MV – methylviologen; OBZ – oxybenzone; OJIP curve – the chlorophyll *a* fluorescence transient; *p*-BQ – p-benzoquinone; PEA – plant efficiency analyzer; PET – photosynthetic electron transport; P_N – net photosynthetic rate; PPCPs – pharmaceuticals and personal care products; PQ – plastoquinone; ROS – reactive oxygen species; RuBP – ribulose-1,5-bisphosphate; V_J – variable fluorescence at 'J' point.
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plants *via* their roots remains unresolved. Moreover, the degree to which OBZ can harm cultivated plants is unclear. By investigating the effects of different OBZ contents on the growth, Chl content, Chl fluorescence transient and PET of PSI, PSII, and whole linear electron transport of the cucumber leaves, we aimed to answer these questions. Answering these questions could help us further understand the negative effect of OBZ on plants and its mechanism and provide further evidence to avoid the use of sunscreen or PPCPs containing OBZ in order to protect the environment and maintain the balance of marine and terrestrial ecosystems.

Materials and methods

Plant materials: A cucumber (*Cucumis sativus* L.) cultivar 'Jinyou-35' was used as the experimental material. The research was done in Tai'an, China. The plant seedlings were cultured in pots filled with culture substrate. Sufficient water and nutrients were supplied throughout the growth period. The growing conditions were the same as that described in our previous research (Zhong *et al*. 2019b). The cucumber seedlings were irrigated with nutrient solution containing different OBZ contents (0, 2.28, 22.8, and 45.6 mg L^{-1}) every morning. To rinse off the OBZ accumulated the day before, the seedlings were watered with the nutrient solution, the volume of which was twice the maximum water content of the soil to ensure that the OBZ content was the same throughout the growing period.

OBZ suspensions: The OBZ was bought from *Sigma Aldrich Company*. The preparation and preservation of OBZ suspensions were performed according to Zhong *et al*. (2019a). The OBZ solids were first dissolved in a small amount of alcohol (0.5 mL) and then put into the Hoagland nutrient solution or reaction agents to make the suspensions with different contents used in this experiment. Hoagland nutrient solutions or reaction agents without OBZ were used as control treatments. The control solution and reaction agent were prepared in exactly the same way as the other OBZ suspensions, except without OBZ.

Plant growth: The height, leaf area, stem diameter, and Chl content of the cucumber seedlings were measured after the seedlings were treated with different OBZ contents for 15 d. The main stem height was measured as a plant height. The stem diameter, at 1 cm above the base, was measured using a micrometer. The leaf area was measured using a *CI-202* leaf area meter (*CID Bio-Science Inc.*, Washington, USA). The Chl content was measured according to Porra (2002). Leaf chlorophyll was extracted with 80% acetone in the dark for 72 h at room temperature. The extracts were analyzed using UV-visible spectrophotometer *UV-1601* (*Shimadzu*, Japan) to measure the OD values at 663 and 645 nm.

Gas exchange: After 15 d of OBZ treatment, the measurement of gas exchange was performed using a *CIRAS-3* photosynthesis system (*PP-Systems*, Hitchin, USA) under ambient CO_2 concentration (400 μmol mol^{-1}), saturated light intensity [1,000 μmol(photon) m^{-2} s^{-1}], room temperature (25°C), 60% relative humidity, all controlled by *CIRAS-3*. All the measurements were performed between 08:00–11:00 h on sunny days. The P_N-C_i response curve was made according to Zhong *et al*. (2019a). The carboxylation efficiencies (CE) were computed on the base of initial slope of the P_N-C_i response curves. The ribulose-1,5-bisphosphate (RuBP) maximal regenerating rates were the maximal P_N under a saturation CO_2 concentration and saturation light intensity (Farquhar *et al*. 1980).

Chl fluorescence transient and 820-nm reflection curve: The Chl fluorescence transients (OJIP curves) and 820-nm reflection curves were synchronously measured using an *M-PEA* instrument (*Hansatech*, King's Lynn, UK) according to Strasser *et al*. (2000). The leaves were cut into discs with 0.38 cm^2 area; the discs were put into the centrifuge tube with different OBZ suspensions and then vacuum-treated for about 15 min to make sure that OBZ had entered leaf discs. The leaf discs were later dark-adapted for about 30 min. The OJIP and 820-nm reflection curves of dark-adapted discs were detected by illuminating with 1-s saturated light [5,000 μmol(photon) m^{-2} s^{-1}] provided by *M-PEA*.

Assay of PET: Chloroplast thylakoid membrane was extracted according to Zhong *et al*. (2019a), and the PET activities were measured using an *Oxytherm* oxygen electrode system (*Hansatech*, King's Lynn, UK). The activity of the whole PET chain was detected as O_2 consumption with addition of 0.1 mM methylviologen (MV) (Brandle *et al*. 1977). The PSII and PSI activities were detected according to Satoh *et al*. (1992) and De la Torre and Burkey (1990). The preparation of the reaction solution of control and OBZ treatment and the calculation of the inhibition ratio of electron transport by OBZ were performed according to Zhong *et al*. (2019a).

Statistical analysis: Least significant difference was computed with *SPSS 11* program (*SPSS Inc*., Chicago, IL, USA). Standard error (SE) was computed with *Excel* (*Microsoft*, Redmond WA, USA).

Results

Plant growth: Irrigation with different OBZ concentrations obviously suppressed the growth of cucumber seedlings (Fig. 1*A*). The plant height, stem diameter, leaf area, and leaf Chl content were significantly lower in OBZ-treated cucumber plants than that in control plants. Treatment with different OBZ contents showed different inhibition extents on plants growth (Fig. 1).

Photosynthetic gas exchange: OBZ significantly inhibited the P_N and g_s of cucumber leaves under saturating light intensity, and these inhibitions intensified as the OBZ content increased. The P_N of cucumber leaves decreased by approximately 57% after the 45.6 mg L^{-1} OBZ treatment. Although the g_s decreased in similar ways as the P_N did, the C_i did not change significantly with the increase in

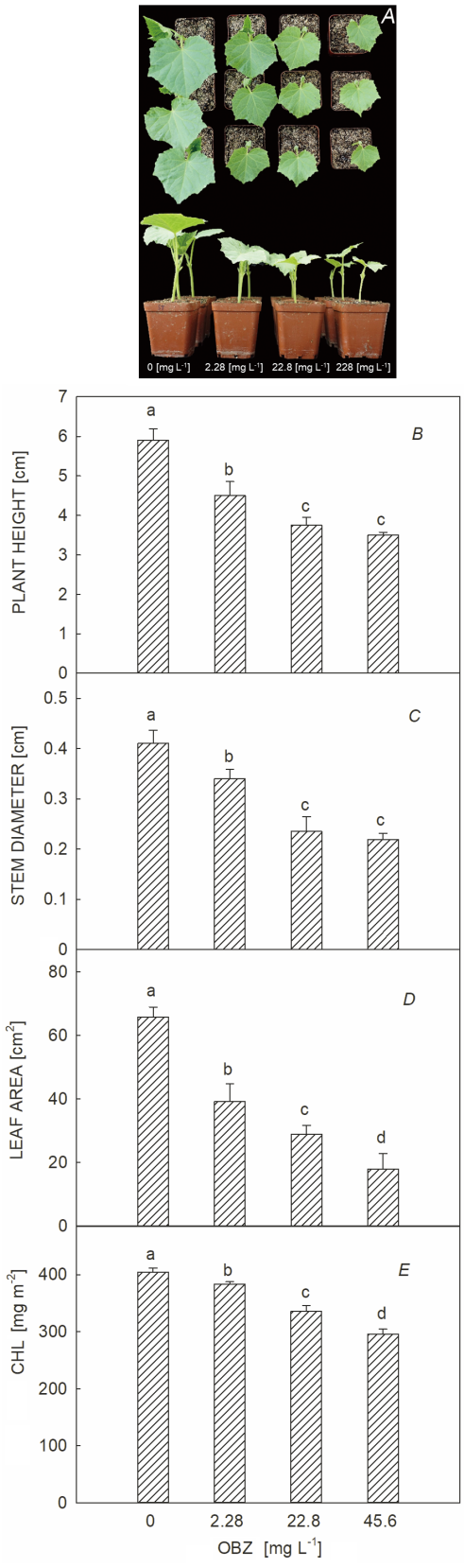

Fig. 1. The effects of 15-d treatments with different oxybenzone contents on the growth (*A*), plant height (*B*), stem diameter (*C*), leaf area (*D*), and chlorophyll content (*E*) of cucumber.

OBZ content. To further analyze photosynthetic CO_2 assimilation, the CO_2-response curves were measured under saturating light intensity. Irrigation with OBZ decreased both the CE and RuBP maximum regeneration rate in cucumber leaves (Fig. 2).

Chl fluorescence transients and 820-nm reflection curves: In order to investigate the mechanism of OBZ inhibition, we used suspensions with different OBZ content to treat leaf discs for a short period of time (45 min). The OJIP curves and the normalized 820-nm reflection curves were significantly affected by different-OBZ-content suspensions. The changes in the Chl fluorescence curve (V_t) indicated that the inhibition ratio of PSII activity by OBZ increased with increasing OBZ content. The 'J' point in the OJIP curve increased sharply with a rise of OBZ content (Fig. 3*A*). The 'J' point rose to the same height as the 'P' point (Fig. 3*A*) when seedlings were treated with the 45.6 mg L^{-1} OBZ suspension. The 820-nm reflection signals started declining soon after turning on the red light but after approximately 20 ms, the decrease in 820-nm reflection was replaced by an increase in reflection. However, after treatment with suspensions of diverse OBZ content, the reversal of the 820-nm reflection was inhibited, and the inhibition of the reversal increased with the increasing OBZ content. After treatment with the 45.6 mg L^{-1} OBZ suspension, the reversal was almost completely blocked (Fig. 3*B*), and it decreased further beyond this point.

PET of the chloroplasts: Based on measuring partial reactions of the full PET chain, OBZ was found to significantly inhibit the whole chain linear PET ($H_2O \rightarrow$ MV) but not electron transfer through PSII or PSI alone, and the degree of inhibition increased with the increase in the OBZ content. The whole chain of PET was inhibited by approximately 92% by the 45.6 mg L^{-1} OBZ. However, neither the low OBZ content nor high OBZ content inhibited the activity of PSII [$H_2O \rightarrow$ *p*-benzoquinone (*p*-BQ)] and PSI [2,6-dichlorophenolindophenol (DCPIP) → MV] (Fig. 4).

Discussion

This study demonstrated that OBZ particles can be absorbed by plant roots even though the particles are insoluble in water. Suspensions with OBZ significantly restrained the P_N and growth of the plant (Figs. 1, 2). Although P_N and g_s decreased with increasing OBZ in a similar manner (Fig. 2*A*,*B*), no significant change in C_i was observed with increasing OBZ (Fig. 2*C*). According to the criteria proposed by Farquhar and Sharkey, a decrease in P_N is caused by stomatal limitation only when g_s and C_i decrease in a similar way, otherwise, such a decrease is caused by nonstomatal factors (Farquhar and Sharkey 1982). Therefore, the inhibition of photosynthesis in cucumber plants by OBZ was caused by nonstomatal factors, which include the diffusion resistance of CO_2 in the mesophyll, light absorption and conversion, and PET and CO_2 assimilation through the Calvin-Benson cycle.

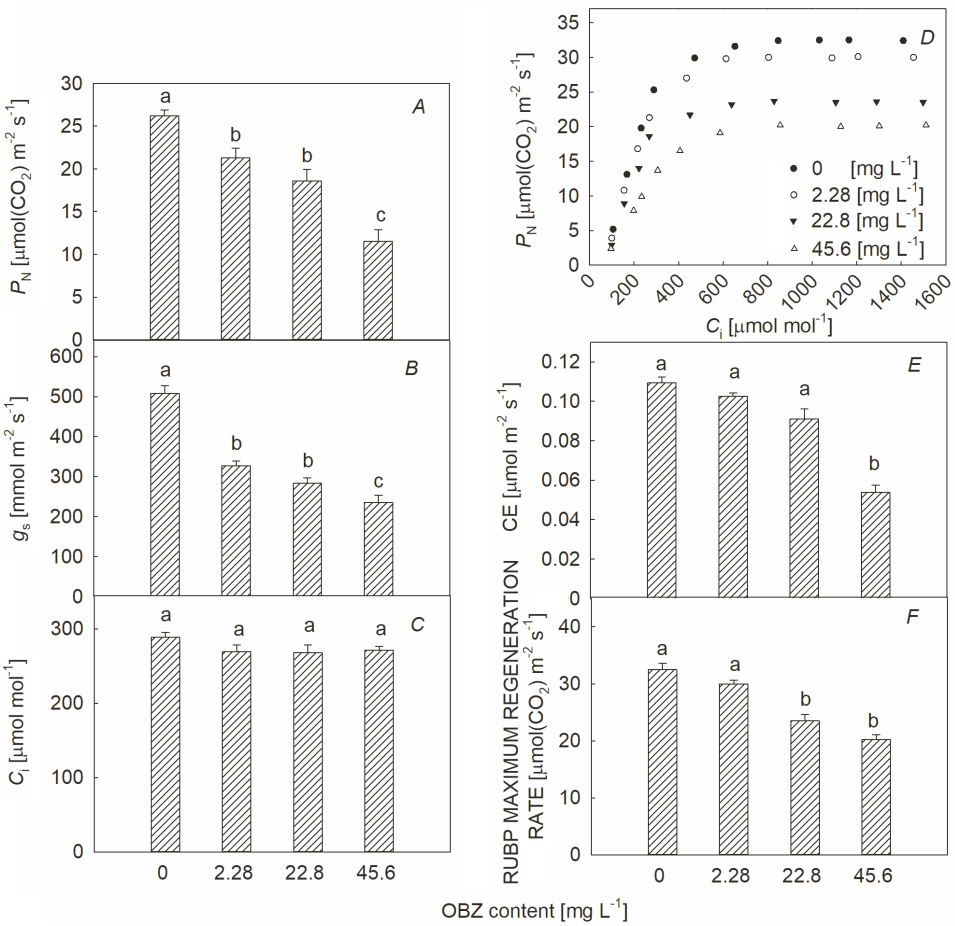

Fig. 2. The effects of 15-d treatments with different oxybenzone contents on the photosynthetic rate (P_N) (*A*), stomatal conductance (g_s) (*B*), intercellular CO_2 concentration (C_i) (*C*), P_N-C_i response curve (*D*), carboxylation efficiency (CE) (*E*), and ribulose-1,5-bisphosphate (RuBP) maximal regenerating rates (*F*) in cucumber plants.

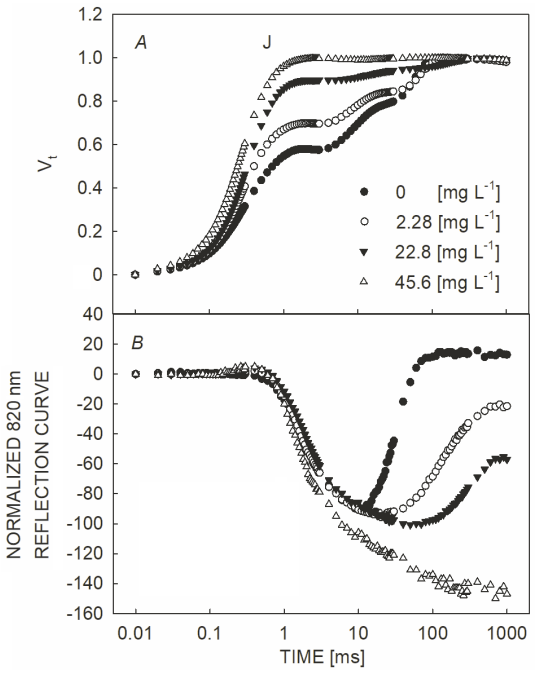

Fig. 3. The effects of 30-min treatments with different oxybenzone contents on the OJIP curves of leaf discs. V_t curve [relative variable fluorescence at any time, $V_t = (F_t - F_0)/(F_m - F_0)$] (*A*) and normalized 820-nm reflection curve (*B*).

To explore the mechanism of photosynthesis inhibition by OBZ, we analyzed the influence of OBZ on the Chl fluorescence transient, 820-nm reflection curve, and PET of PSI, PSII, and the whole linear electron transport chain as well as the P_N-C_i response curve, CE and RuBP maximal regenerating rates of photosynthesis.

The Chl fluorescence transient has been widely used as a powerful tool to assess photosynthesis. The OJIP test established by Strasser (1978) has been used efficiently to reflect PSII activity (Jiang *et al*. 2006, Strasser and Stirbet 2001, Fan *et al*. 2014). A large number of studies have proven that an increase in the 'J' point of the fluorescence transient indicates severe inhibition of the linear electron transport chain of photosynthesis from Q_A to downstream electron receptors (Haldimann and Strasser 1999, Jia *et al*. 2010). When electron transport is completely blocked by 3-(3,4-dichlorophenyl)-1,1-dimethyl-urea (DCMU), which inhibits electron flow from Q_A to the Cyt b_6f complex, the 'J' point reaches a level as high as that of the 'P' point (Schansker *et al*. 2003). Therefore, the observation that the 'J' point reached the 'P' point after treatment with a 45.6 mg L^{-1} OBZ suspension indicated that the treatment almost completely blocked the electron transport from Q_A to the Cyt b_6f complex.

In the past decade, the 820-nm absorption (Schansker *et al*. 2003) or reflection (Zhang *et al*. 2011) of leaves or chloroplasts has been used to reflect the redox activity

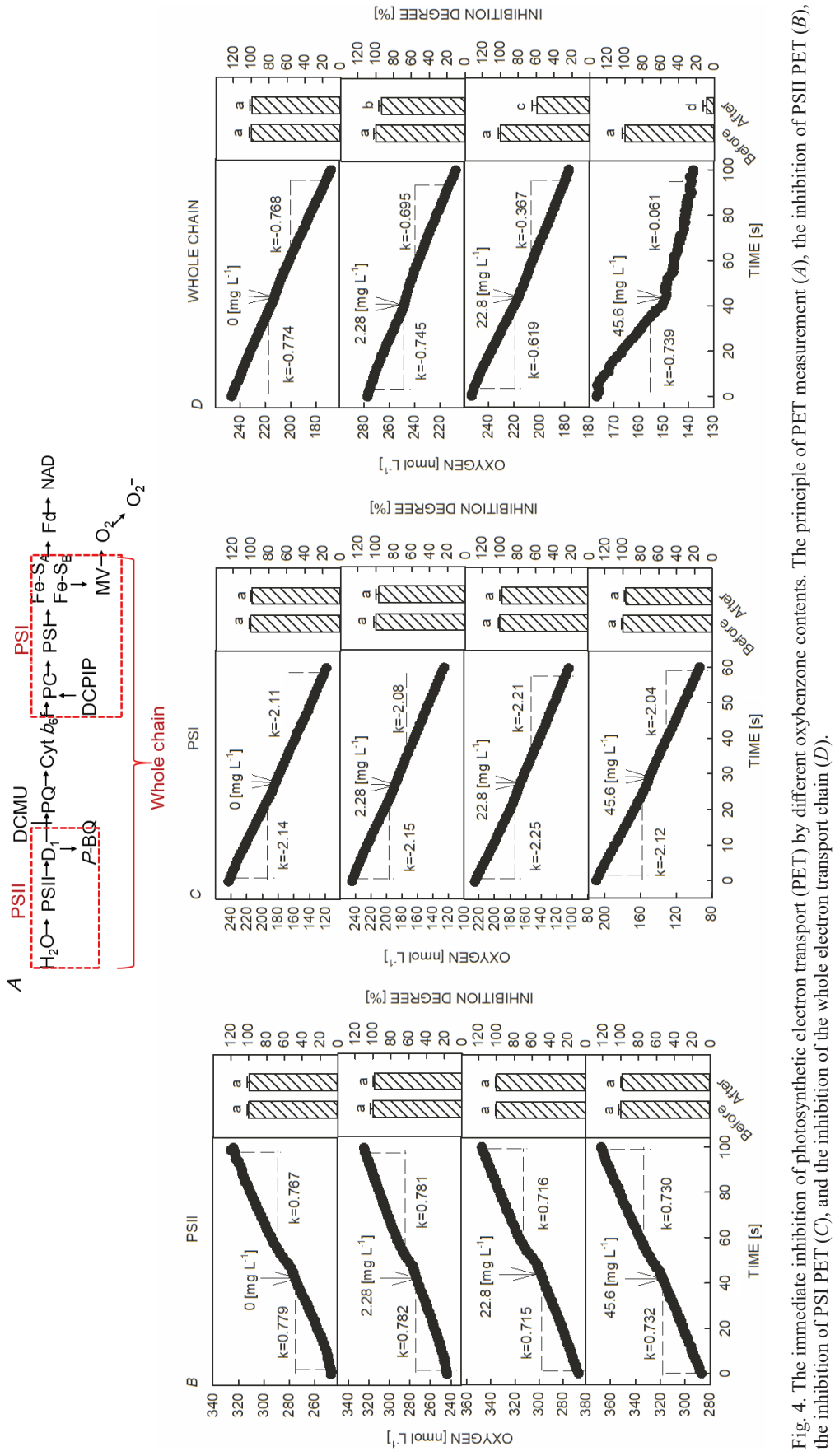

Fig. 4. The immediate inhibition of photosynthetic electron transport (PET) by different oxybenzone contents. The principle of PET measurement (*A*), the inhibition of PSII PET (*B*), the inhibition of PSI PET (*C*), and the inhibition of the whole electron transport chain (*D*).

of PSI. A reversal of the decrease in 820-nm reflection is caused by the arrival of electrons from PSII (Schansker *et al*. 2003). The inhibition of the reversal of 820-nm reflection indicated that the electrons sent from PSII to reduce PSI were blocked. The fact that the reversal of 820-nm reflection was almost completely blocked by the 45.6 mg L^{-1} OBZ suspension further supported the assumption discussed above, specifically, that the electron transport from Q_A to the Cyt b_6f complex was almost completely blocked by 45.6 mg L^{-1} OBZ, so that PSI could not be reduced by electrons from PSII.

Analysis of the OJIP test and the changes in 820-nm reflection indicated that the inhibition of electron transport from Q_A to the Cyt b_6f complex was the principal reason for OBZ inhibition of photosynthesis. To further confirm this conclusion and narrow the hypothesized inhibition site, we used a more direct method of detecting O_2 release (PSII) or O_2 consumption (PSI and the whole linear chain) to evaluate the electron transport capacities of PSI, PSII, and the whole linear chain in chloroplasts with and without OBZ treatments.

OBZ did not inhibit the activity of PSII ($H_2O \rightarrow p$-BQ) and PSI (DCPIP → MV) but significantly inhibited the whole linear electron transport chain ($H_2O \rightarrow$ MV) (Fig. 4*B–D*), confirming that OBZ inhibited PET by inhibiting PQ and/or the Cyt b_6f complex between PSI and PSII. Blocking PET in PQs and/or the Cyt b_6f complex restricts the transport of the electrons generated by H_2O photolysis in PSII to PSI, resulting in the accumulation of a large number of electrons in PSII reaction centers and, consequently, closure of these reaction centers. This hypothesis was supported by the sharp increase in the 'J' point of the OJIP curve (Fig. 3*A*). The inhibition of PET leads to an increase in excess excitation energy, which inevitably causes overproduction of ROS (Zhang *et al*. 2014, Zhong *et al*. 2019a), further damaging the photosynthetic apparatus. We think this process underlies the indirect inhibition of photosynthesis by OBZ.

The inhibition of PET limits the synthesis of NADPH and ATP. ATP plays an important role in regulating the activity of Rubisco (EC 4.1.1.39), a key enzyme for CO_2 assimilation. The actual Rubisco activities depend greatly on Rubisco-activase activity (Portis 2003). ATP is involved in the activation of Rubisco (Zielinski *et al*. 1989, Portis *et al*. 2008). The decrease in ATP synthesis caused by OBZ reduced Rubisco activation and CE. Furthermore, limiting the synthesis of NADPH and ATP directly inhibits CO_2 reduction and RuBP regeneration through the Calvin-Benson cycle, which was supported here by the fact that the CE and RuBP maximal regenerating rates were significantly inhibited by OBZ treatment (Fig. 2*D*). Therefore, the decrease in CO_2 assimilation was mainly due to the inhibition of PET by OBZ.

OBZ can enter rivers, lakes, swimming pools, groundwater, sewage, and irrigation water *via* human activities (Li *et al*. 2007, Zwiener *et al*. 2007, Goksøyr *et al*. 2009, Sánchez Rodríguez *et al*. 2015). Schneider and Lim (2019) found that these ultraviolet filters, including OBZ, are difficult to remove using conventional sewage treatment. The purpose of this study was to explore the OBZ inhibition mechanism. In order to shorten treatment time and enhance the inhibition extent, we used higher OBZ content (over 2.28 mg L^{-1}) in this study. In our previous study, we have proved that lower OBZ content such as 0.228 mg L^{-1} could obviously inhibit PET (Zhong *et al*. 2019a). Downs *et al*. (2016) have found that the detected OBZ content was 75 μg L^{-1}–1.395 mg L^{-1} in the Virgin Island, which is much higher than the content we have tested previously. In arid and semiarid regions, the reuse of treated wastewater for agricultural irrigation is increasing, and OBZ is thus more likely to be transferred to agricultural land (Wu *et al*. 2015). In our study, OBZ significantly affected the photosynthesis and growth of a higher plant through the irrigation water. Other scientists have also reported that biosolids in PPCPs are absorbed by plants *via* the roots and then accumulate continuously in plants (Wu *et al*. 2010, Cabrera-Peralta and Peña-Alvarez 2018). The results of our study can help us understand the limitation of agricultural production caused by OBZ and provide evidence to support limitations for the use of OBZ. The results also suggest the potential impact of other biosolids on agricultural production.

Our research demonstrated that the inhibition of photosynthesis in cucumber leaves by OBZ mainly resulted from the blocking of PET and that the possible PET inhibition sites were PQs and/or the Cyt b_6f complex. To locate the precise inhibition site (PQs or Cyt b_6f or both of them) needs more research. And it is worth clarifying the inhibition mechanism of PQ and/or Cyt b_6f complex

Conclusions: Our research demonstrated that OBZ could be absorbed by plant roots and significantly inhibited photosynthesis and growth of cucumber seedlings. OBZ directly blocked linear PET by inhibiting PQs and/or Cyt b_6f complex, restricting the reduction of PSI reaction center. The inhibition of PET can cause accumulation of ROS and lack of assimilatory power (ATP and NADPH), which would damage cell membrane and photosynthetic apparatus, and restrict CO_2 assimilation *via* inhibiting the Calvin-Benson cycle. Therefore, it is important to limit the use of sunscreens containing OBZ to protect aquatic and terrestrial ecosystems and ensure the safety of agricultural production.

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