Effect of Mycotoxin Cytochalasin A on Photosystem II in Ageratina adenophora

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Abstract: Biological herbicides have received much attention due to their abundant resources, low development cost, unique targets and environmental friendliness. This study reveals some interesting effects of mycotoxin cytochalasin A (CA) on photosystem II (PSII). Our results suggested that CA causes leaf lesions on Ageratina adenophora due to its multiple effects on PSII. At a half-inhibitory concentration of 58.5 µM (I_{50}, 58.5 µM), the rate of O_2 evolution of PSII was significantly inhibited by CA. This indicates that CA possesses excellent phytotoxicity and exhibits potential herbicidal activity. Based on the increase in the J-step of the chlorophyll fluorescence rise OJIP curve and the analysis of some JIP-test parameters, similar to the classical herbicide diuron, CA interrupted PSII electron transfer beyond Q_A at the acceptor side, leading to damage to the PSII antenna structure and inactivation of reaction centers. Molecular docking model of CA and D1 protein of A. adenophora further suggests that CA directly targets the Q_B site of D1 protein. The potential hydrogen bonds are formed between CA and residues D1-His215, D1-Ala263 and D1-Ser264, respectively. The binding of CA to residue D1-Ala263 is novel. Thus, CA is a new natural PSII inhibitor. These results clarify the mode of action of CA in photosynthesis, providing valuable information and potential implications for the design of novel bioherbicides.

Keywords: natural product; bioherbicide; photosynthetic inhibitor; chlorophyll a fluorescence; D1 protein; molecular docking

1. Introduction

Weed control is always a great concern in agriculture due to its deduction in crop yields. In the last decades, chemical herbicides have been widely used to control the growth of weeds. However, some chemical herbicides have not been effective in controlling weeds due to the prevalence of resistant weeds because of long-term use [1]. In addition, chemical herbicides are polluting the environment and causing many negative effects on living organisms including human. These reasons have prompted attention to the development and application of novel bioherbicides which are more environmentally friendly. Natural compounds are extensively used in the development of bioherbicides. Several studies have shown that natural compounds derived from plants and microorganisms are effective in controlling weed seed germination and growth [2–4], becoming an important resource for the discovery of new natural herbicidal components and action targets.

Cytochalasins are a class of natural products produced by molds, including cytochalasin A, B, C, D, E, F, etc. More than one hundred cytochalasin-related structures or their derivatives have been identified and reported from molds like Chaetomium, Rosellinia, Zygosporium, Helminthosporium, Penicillium, Aspergillus, etc. [5]. In terms of structure, cytochalasins are typically characterized by a substituted perhydro-isoindolone moiety derived from a highly reduced polyketide backbone and amino acids, attached to a macrocyclic ring [5,6]. As for cytochalasin A (CA), the structure is shown in Figure 1A. It is worth...
mentioning that CA is an oxidized derivative of cytochalasin B (CB), and both differ only in the group attached at C-20 [7], which display as the carbonyl group and the hydroxyl group, respectively. Because of the structural similarities between cytochalasins, they have analogous biological activities. There are numerous studies on the effect of cytochalasins on animal cells. Cytochalasins are most well-known for targeting microfilaments [8] and thereby inhibiting various cellular processes, including actin polymerization [9–11], platelet agglutination [12], glucose transport [13,14], mitochondrial contraction [15], etc. In addition, the antitumor [16,17] activity of cytochalasins was also reported.

![Chemical structure of cytochalasin A](image)

**Figure 1.** (A) Chemical structure of cytochalasin A (C29H35NO5, MW = 477.6). (B) Lesion formation of *A. adenophora* leaves treated with mock (10% acetone) and 100, 200, 400 and 800 μM cytochalasin A. (C) Effect of cytochalasin A on oxygen evolving rate of *C. reinhardtii* cells. Data shown are mean ± SE of three independent measurements with around 15 repetitions. The different lowercase letters (a, b, c, d, e) indicate to be statistical significance at 0.05 level.

For the study of cytochalasins in plant cells, it has been reported that because of the action of cytochalasins on the actin cytoskeleton, they inhibit the motility of various organelles in plant cells, including chloroplasts [18,19], mitochondria [20], endoplasmic reticulum [21], Golgi stacks [22,23] and peroxisomes [24]. The disruption of the actin cytoskeleton by cytochalasins leads to activation of the salicylic acid pathway to enhance plant resistance to pathogens [25,26]. In addition, cytochalasin B and F were reported to be effective in inhibiting root elongation of wheat and tomato seedlings [27], showing an interesting herbicidal activity. Photosynthesis, one of the most critical physiological processes in green plants and the main target of many commercial herbicides, is also influenced by cellular cytochalasins. Several cytochalasins, including CA, have been shown to have the ability to inhibit photosynthesis. CA and CB reduced the light absorption capacity of the leaves, resulting in the suppression of photosynthetic efficiency [28]. Cytochalasin F inhibited the photosynthetic activity of Green Alga *Chlorella fusca* and was shown to be an inhibitor of photosynthesis [29]. Cytochalasin E was found to have a direct effect on photosynthesis by chlorophyll (Chl) fluorescence assay, probably due to the impairment of light harvesting [30]. In summary, we believe that cytochalasins are phytotoxic and have potential bioherbicidal activity. Thus, as a member of the cytochalasin family and being capable of inhibiting photosynthesis, CA’s mechanism of action in photosynthesis is highly inspiring for the development of innovative natural herbicides.

Although there are several reports on the effects of CA in plants, the specific mechanism of action of CA in plant cells is unclear, especially the target of photosynthesis inhibition. In photosynthetic reactions, photosystem II (PSII) is considered as the crucial photosynthetic component, catalyzing light-driven water-splitting and oxygen-evolving
reaction, thereby converting light energy into electrochemical potential energy and generating molecular oxygen [31]. As is well known, many classical herbicides target PSII in competition with the native electron acceptor plastoquinone, binding at the Q_B site of the D1 protein, thereby blocking the transfer of electrons from Q_A to Q_B to inhibit photosynthesis [32,33]. Q_A and Q_B are the primary and secondary plastoquinone receptors in PSII, respectively. The highly conserved amino acid residues in the Q_B site may play a key role in the binding to PSII inhibitors in plants [34], and molecular docking studies have been reported in plants to identify the binding properties of some widely used commercial PSII inhibitory herbicides to the Q_B site within the D1 protein [34]. The molecular details of the interaction of many natural products possessing PSII inhibitory effects in the D1 Q_B site in plants remain to be elucidated. Therefore, it is important to explore the specific effects and sites of the natural compound CA on photosynthesis for the development of novel bioherbicides in the future.

In this work, we treated the leaves of Ageratina adenophora with CA and found that CA can cause leaf diseases. In addition, the oxygen evolution rate and photosynthetic activity of Chlamydomonas reinhardtii were reduced by CA. From these results, we inferred that CA has great potential for weed management due to its phytotoxicity and ability to inhibit PSII photosynthetic activity. Based on the study of Chl a fluorescence rise kinetics and the analysis of molecular docking, we aimed to determine the mode of action and the site of action of CA in PSII. It was found that CA interrupts PSII electron transfer beyond Q_A at the acceptor side, leading to impaired PSII antenna structure and inactivation of the reaction centers. The interaction model between the CA and D1 proteins of A. adenophora was simulated to discover the accurate binding site. The revelation of the effect of CA on PSII and the specific binding sites of CA in PSII probably helps to better utilize it as a tool for plant research, especially in the development of new effective photosynthetic herbicides.

2. Results and Discussion

2.1. CA Caused Leaf Lesions in A. adenophora and Reduced the Rate of Oxygen Evolution in C. reinhardtii

CA, a fungal phytotoxin, had an inhibitory effect on the growth and viability of poplar suspension-cultured cells because the dry weight and oxygen consumption of cells treated with CA were strongly inhibited [35]. To estimate the phytotoxicity of CA, the damage formation in mature leaves of A. adenophora after treatment with different concentrations of CA was monitored. The monitored lesion formation is shown in Figure 1B. At the highest concentration of 800 µM, the largest diameter of visible lesions on the leaves was observed, with necrosis and browning of the leaves in the treated area. The formation of necrosis or chlorosis implies severe damage to photosynthetic tissues from chlorophyll breakdown [36]. This suggested that CA possibly impaired the photosynthetic activity of A. adenophora leaves.

To verify the effect of CA on photosynthetic apparatus, we measured the PSII oxygen evolution rate of C. reinhardtii cells incubated in different concentrations of CA. At a CA concentration of 100 µM, the rate of oxygen evolution was significantly reduced by approximately 64% (Figure 1C). The calculated \( I_{50} \) value (concentration required for 50% inhibition) was 58.5 µM. Diuron (3-(3′,4′-dichlorophenyl)-1,1-dimethylurea, DCMU), a classical PSII inhibitor, has been reported to completely inhibit the evolution of oxygen in C. reinhardtii cells at 1 µM [37] with an \( I_{50} \) value of 0.12 µM [38]. This suggests that CA has a good inhibitory effect on PSII, but is a weaker PSII inhibitor compared with diuron. In fact, diuron is a strong and specific PSII inhibitor, compared with which most natural photosynthetic inhibitors are less active [38]. It has been shown that the phytotoxin tenuazonic acid (TeA) and gliotoxin inhibit the rate of oxygen evolution with \( I_{50} \) values of 261 µM and 60 µM, respectively [36,39]. However, there are also stronger natural PSII inhibitors, such as sorgoleone, which have a similar ability to inhibit photosynthetic \( O_2 \)
evolution as diuron [40]. Thus, CA has an inhibitory effect on photosynthesis in plants and is a weaker PSII inhibitor.

2.2. CA Inhibited Photosynthesis Activity of C. reinhardtii

Chl a fluorescence is very sensitive to stress-induced changes in photosynthesis. To further demonstrate the inhibition of photosynthetic activity by CA, the changes in fluorescence parameters of CA-treated C. reinhardtii cells were monitored by Imaging-PAM fluorometer. Figure 2A shows the color-coded images of F0, Fm and Fv/Fm. F0, minimal fluorescence from dark-adapted leaf samples, indicates the level of fluorescence when QA is maximally oxidized, at which point the PSII centers are completely open [41]. Correspondingly, Fm indicates the level of fluorescence when QA is maximally reduced, at which point the PSII centers closed [41]. F0 usually represents phototropic pigment activity [42]; the decrease in Fm may be correlated with the quenching of fluorescence due to the oxidation of the plastoquinone pool or the damage of the PSII antennae [43]. Fv/Fm represents the PSII maximum quantum yield. Under different concentration of CA treatments, images of F0 remained relatively stable, while images of Fm faded gradually. Under the treatment of CA, the Fv/Fm image fades from dark blue to light blue. The values of the fluorescence parameter Fv/Fm (Figure 2B) were slightly reduced but not significantly different. Electron transfer rate (ETR), a more sensitive [44] fluorescence parameter, showed significant concentration-dependent changes, with values decreasing with increasing CA concentration (Figure 2C). At the highest concentration of 40 μM CA, the ETR value was close to 0. The parameter values of ETR decreased approximately linearly, indicating that the inhibition of PSII electron transfer should be an important action site for CA on the photosynthetic apparatus.

![Figure 2](image-url)  
**Figure 2.** Effects of cytochalasin A on color fluorescence imaging of F0, Fm and Fv/Fm (A), the value of the maximum quantum yield of PSII (Fv/Fm) (B), and electron transport rate (ETR) (C) of C. reinhardtii cells. Fluorescence images were indicated by color code in the order of black (0) through red, orange, yellow, green, blue, violet to purple (1). The number codes above images are marked from 0 to 1, showing the changes. Each value is the average ± SE of three independent experiments with around 15 repetitions. The different lowercase letters (a, b, c, d) indicate to be statistical significance at 0.05 level.

2.3. Fast Chl a Fluorescence Rise Kinetics OJIP of CA-Treated A. adenophora

For decades, Chl fluorescence rise kinetics has been widely used in studies of the structure and function of the photosynthetic apparatus because of its advantages of being fast, non-invasive and precise [41]. To further probe the accurate site of action of CA on PSII, we measured the fluorescence rise OJIP curves of CA-treated A. adenophora leaf discs (Figure 3). As shown in Figure 3A, the fluorescence rise curve of the control exhibited a typical polyphasic O-J-I-P shape. The fluorescence intensity increases from F0 (O-step) to Fm (P-step) and through two intermediate steps, Fj (J-step) and F1 (I-step). The initial fluorescence F0 at 20 μs, and the intermediate basic fluorescence data Fj and F1 at 2 and
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30 ms, respectively. \( V_t \), representing relative variable fluorescence at time \( t \), is calculated with the formula \( V_t = (F_t - F_0)/(F_M - F_O) \). The detailed parameters and definitions were listed in Table 1, which is according to Strasser et al. [41] and Chen et al. [45]. Under CA treatment, the shape of the fluorescence rise transient curve changed remarkably. As the concentration of CA and treatment time increased, the \( F_M \) decreased significantly and the I- and P-steps gradually disappeared (Figure 3A,C). The decrease of \( F_M \) is generally related to increased fluorescence quenching and the damage of antenna pigment [43]. Here, we proposed that CA affected the structure of antenna pigment so as to decrease the photosynthesis capacity.

Figure 3. Effects of cytochalasin A on fast chlorophyll a fluorescence rise kinetics OJIP of A. adenophora leaf discs. (A,B) Raw Chl a fluorescence rise kinetics and its variable changes normalized by \( F_0 \) and \( F_M \) as \( V_t = (F_t - F_0)/(F_M - F_O) \) (top) and \( \Delta V_t = V_t(treatment) - V_t(control) \) (bottom) in a logarithmic time scale of A. adenophora treated with different concentrations (0, 25, 50, 100 and 200 µM) of cytochalasin A for 12 h. (C,D) Raw Chl a fluorescence rise kinetics and its variable changes normalized by \( F_0 \) and \( F_M \) as \( V_t = (F_t - F_0)/(F_M - F_O) \) (top) and \( \Delta V_t = V_t(treatment) - V_t(control) \) (bottom) in a logarithmic time scale of A. adenophora treated with 50 µM cytochalasin A for different time periods (0, 3, 6 and 12 h). Each curve is the average of three independent experiments with around 15 repetitions.

To explore the information behind OJIP curves, the fluorescence data were double-normalized by \( F_0 \) and \( F_M \), as relative variable fluorescence \( V_t = (F_t - F_0)/(F_M - F_O) \) (top) and \( \Delta V_t = V_t(treatment) - V_t(control) \) (bottom) on logarithmic time scale (Figure 3B,D). Clearly, CA led to a concentration- and time-dependent rise of J-step, which is a classical phenomenon of the blockage of electron flow beyond the \( Q_A \) at the PSII acceptor side and the large accumulation of \( Q_A^- \) [41]. Treatment of A. adenophora leaf discs with the specific PSII inhibitor diuron resulted in a rapid rise in J-step to the same \( F_M \) level at a concentration of 100 µM [38]. Apparently, the effect of CA on the kinetics of fluorescence rise is similar to that of diuron, mainly causing a significant increase in the J-peak.

The elevated level of the J-step and the decrease in \( F_M \) suggest that CA not only blocked PSII electron flow beyond the \( Q_A \), but also impaired the structure and function of the PSII antenna.
### Technical Fluorescence Parameters

| Parameter | Explanation |
|-----------|-------------|
| \( F_t \) | Fluorescence at time \( t \) after onset of actinic illumination |
| \( F_0 \) | Minimal fluorescence, when all PSII RCs are open |
| \( F_L \) | Fluorescence intensity at the L-step (150 µs) of OJIP |
| \( F_K \) | Fluorescence intensity at the K-step (300 µs) of OJIP |
| \( F_J \) | Fluorescence intensity at the J-step (2 ms) of OJIP |
| \( F_P \) | Maximal fluorescence, at the peak P of OJIP |
| \( F_v \) | Variable fluorescence at time \( t \) |
| \( V_t \) | Relative variable fluorescence at time \( t \) |
| \( V_J \) | Relative variable fluorescence at the J-step |
| \( V_I \) | Relative variable fluorescence at the I-step |

#### Quantum efficiencies or flux ratios

- \( \phi_{Po} = \frac{PHI(P_0)}{TR_0/ABS} = 1 - \frac{F_O}{F_M} \)  
  - Maximum quantum yield for primary photochemistry
- \( \psi_{Eo} = \frac{PSI_0}{ET_0/TR_0} = (1 - V_J) \)  
  - Probability that an electron moves further than QA
- \( \phi_{Eo} = \frac{PHI(E_0)}{ET_0/ABS} = (1 - F_O/F_M)(1 - V_J) \)  
  - Quantum yield for electron transport (ET)

#### Phenomenological energy fluxes (per excited leaf cross-section-CS)

- \( ABS/CS = Chl/CS \)
- \( TR_0/CS = \phi_{Po} \cdot (ABS/CS) \)  
  - Trapped energy flux per CS
- \( ET_0/CS = \phi_{Po} \cdot \psi_{Eo} \cdot (ABS/CS) \)  
  - Electron transport flux per CS
- \( RC/CS = \phi_{Po} \cdot (V_J/M_0) \cdot (ABS/CS) \)  
  - Density of reaction center (QA-reducing PSII reaction center–RC)
- \( Q_A\text{-reducing centers} = (RC/RC_{\text{reference}}) \cdot (ABS/ABS_{\text{reference}}) \)  
  - Fraction of QA-reducing PSII RCs per CS
- \( R_J = (V_{J(\text{control})} - V_{J(\text{treatment})})/(1 - V_{J(\text{control})}) \)  
  - Number of PSII RCs with Q_B-site filled by PSII inhibitor

#### Performance indexes

- \( PI_{ABS} = \frac{\gamma_{RC}}{1 - \gamma_{RC}} \cdot \frac{\phi_{Po}}{1 - \phi_{Po}} \cdot \frac{\psi_{Eo}}{1 - \psi_{Eo}} \)  
  - Performance index for energy conservation from photons absorbed by PSII to the reduction of intersystem electron acceptors

### Table 1. Formulae and explanation of the technical data of the OJIP curves and the selected JIP-test parameters used in this study

1. Subscript “0” (or “o” when written after another subscript) indicates that the parameter refers to the onset of illumination, when all RCs are assumed to be open.
2.4. The JIP-Test Analysis

The JIP-test based on the Theory of Energy Fluxes in Biomembranes is a powerful tool for quantifying the fast Chl fluorescence rise OJIP curve, providing access to the vitality of a photosynthetic sample [41]. To further elucidate the effect of CA on PSII, some functional parameters selected from the JIP-test were analyzed. Definitions and specific formulas for certain parameters are shown in Table 1. Obviously, the increase in CA concentration had little effect on the $F_0$ but substantially reduced the $F_M$ values. The ratio of $F_V/F_M$ showed a decline with the increase in CA treatment concentration (Figure 4A). This result is consistent with the results in Figure 2A,B. This implies that CA has a similar effect on the photosynthetic activity of A. adenophora and C. reinhardtii. We concluded that CA caused damage to the structure and function of the PSII antenna and thus inhibited plant photosynthetic activity.

![Figure 4](attachment:figure4.png)

**Figure 4.** Changes of selected JIP-test fluorescence parameters of A. adenophora leaf discs treated with different concentrations of cytochalasin A. (A) $F_O$, $F_M$ and $F_V/F_M$. (B) $V_I$, $V_J$ and $\Delta V_J$. (C) $\varphi_{Eo}$, $\psi_{Eo}$ and $\varphi_{Po}$. (D) ABS/CS$_0$, TR$_0$/CS$_0$ and ET$_0$/CS$_0$. (E) RC/CS, QA-reducing centers, $R_Q$. (F) $P_{ABS}$ and D.F. Each curve is the average of three independent experiments with around 15 repetitions. The different lowercase letters (a, b, c, d) indicate to be statistical significance at 0.05 level.

The value of $V_J$ (variable fluorescence at J-step) and $\Delta V_J$ increased with increasing CA concentration (Figure 4B). At 200 $\mu$M, the $V_J$ value increased to 0.79, which was more than twice as high as that of the control (0.36). The increase in $V_J$ value indicates that CA blocks PSII electron transport beyond Q$_A$. Additionally, the value of $V_I$ (variable fluorescence at I-step) was 12% higher than that of the control at a CA concentration of 25 $\mu$M, but no obvious change in the value of $V_I$ was observed as the CA concentration increased.

In Figure 4C, a dramatic drop was observed in the parameters $\varphi_{Eo}$ and $\psi_{Eo}$ with a calculated $I_{50}$ value of 83.25 $\mu$M and 103.86 $\mu$M for $\varphi_{Eo}$ and $\psi_{Eo}$, respectively. The $\varphi_{Eo}$
denotes the quantum yield of PSII electron transfer, while $\psi_{Eo}$ shows the probability that a trapped exciton moves an electron into the electron transport chain beyond QA$^-$ [41]. The decrease of $\psi_{Eo}$ and $\psi_{Eo}$ values further supports that CA blocks the PSII electron transport beyond QA. By increasing the CA treatment concentrations, the values of $\psi_{Eo}$ also decreased, suggesting that the maximum quantum yield of PSII primary photochemistry was suppressed by CA.

ABS/CS$_0$ (absorption flux per excited leaf cross section), TR$_0$/CS$_0$ (light energy captured per unit leaf area) and ET$_{0}$/CS$_0$ (electron transport flux per leaf cross section) significantly declined under CA treatment [41] (Figure 4D). ABS/CS$_0$ is available as a measure of average antenna size or chlorophyll concentration, and TR$_0$/CS$_0$ reflects the specific rate of exciton trapped per excited leaf cross-section by open reaction centers [46]. The decrease in ABS/CS$_0$ and TR$_0$/CS$_0$ suggests that CA reduced the concentration of chlorophyll and disrupted the conformation of the antenna pigment assembly, thereby reducing the efficiency of light energy transfer between antenna pigment molecules and from PSII reaction centers. The quantum yield of electron transport is influenced, as shown by the decreases in ET$_0$/CS$_0$. The above results indicate that CA dramatically blocked the energy flux between photosynthetic structural units.

Since CA interrupts PSII electron flow from QA to QB, decreases chlorophyll concentration and disrupts the conformation of the antenna pigment assembly, an inactivation event is expected to occur in the PSII reaction center. The fraction of QA-reducing centers were calculated, QA-reducing centers = $\frac{[\text{RC/CS}]_{\text{treatment}}}{[\text{RC/CS}]_{\text{control}}} \times \frac{[\text{ABS/CS}]_{\text{treatment}}}{[\text{ABS/CS}]_{\text{control}}}$. In Figure 4E, the marked decrease in QA-reducing centers suggests that CA did lead to a rapid closure of PSII reaction centers. A significant reduction in QA-reducing centers certainly results in an increase in non-QA-reducing centers, also known as heat sink centers or silent centers, which are radiators and are frequently used to protect the system from over-excitation and over-reduction, resulting in the generation of ROS [45]. The value of RC/CS$_0$ (the density of QA-reducing reaction centers per excited leaf cross section) also exhibited a similar decreased tendency after CA-treatment. This indicated that CA caused damage to the PSII reaction centers and inhibited the activity of PSII.

It has been shown that the PSII reaction centers would become inactivated in the competitive substitution of the QB binding site in the D1 protein by PSII inhibitors, thus interrupting electron transport [41]. To investigate the binding of CA to the QB site of reaction centers, Rj was introduced to estimate the number of PSII reaction centers at the QB site populated by CA. The fluorescence parameter Rj could be derived from the equation $\text{Rj} = \frac{V_{\text{J(treatment)}} - V_{\text{J(control)}}}{1 - V_{\text{J(control)}}}$ [45]. A visible concentration-dependent increase in Rj was observed after CA treatment (Figure 4E). The rise of Rj means that the increased CA molecules occupy the QB site of D1 protein, thus blocking the electron transfer from QA to QB. Therefore, CA-induced inactivation of the PSII reaction center probably results from the binding of CA to D1 protein.

Pl$_{ABS}$, the performance index on absorption basis, is the most sensitive JIP-test parameter for the different stress treatments. Compared with mock, Pl$_{ABS}$ respectively decreased by 75% (25 $\mu$M), 86% (50 $\mu$M), 89% (100 $\mu$M) and 97% (200 $\mu$M) after 12 h of treatment with different concentrations of CA (Figure 4F). The other parameter DF$_{ABS}$ showed a comparable downward trend. DF$_{ABS}$ is derived from the equation $\text{DF}_{ABS} = \log(\text{Pl}_{ABS})$ and can be defined as the total driving force of photosynthesis in the observed system [41]. These results reflected that the overall photosynthetic activity and efficiency of the leaves showed a concentration-dependent decrease after CA treatment.

Altogether, CA might destroy the structure of antenna pigment (F$_{O}$, F$_{M}$, ABS) and inhibit the energy flux between antenna units (ABS/CS$_0$, TR$_0$/CS$_0$ and ET$_0$/CS$_0$) as well as electron transport above QA ($\varphi_{Eo}$ and $\psi_{Eo}$) by binding to the QB binding site of D1 protein (Rj) and thus lead to the dramatic decrease of overall photosynthesis activity (Pl$_{ABS}$) in A. adenophora. To verify the primary site of action of CA on PSII, the correlations between some selected parameters are shown in Figure 5. As shown in Figure 5A, the data displayed a highly linear relationship between F$_M$ and ABS/CS$_0$ or TR$_0$/CS$_0$ in the leaves treated with
different concentrations of CA. It suggested that the CA-caused decrease of $F_M$ was induced by the concentration-dependent decrease of ABS/CS$_0$ or TR$_0$/CS$_0$. So, the inhibition of CA on the efficiency of light energy transfer between antenna pigment molecules and from PSII reaction centers was the main reason for the decrease in $F_M$. $V_{J}$ showed a high negative correlation with $\varphi_{Eo}$ and a high positive correlation with $R_J$ (Figure 5B), which suggested that CA occupied the QA sites in D1 protein to block the PSII electron transport beyond QA, thus leading to a large accumulation of $Q_A^-$ as an increase of $V_{J}$. In addition, the linear relationship between the parameters $R_J$ and ET$_0$/CS$_0$, $\varphi_{Eo}$ and $\psi_{Eo}$ further demonstrated that CA interferes with electron transport by occupying the QA-binding site (Figure 5C). Under CA treatment, the decrease of the parameter QA-reducing centers was correlated with ET$_0$/CS$_0$ and $\psi_{Eo}$, respectively (Figure 5D). It indicated that CA caused the interruption of PSII electron transport beyond the QA, which resulted in the inactivation of the reaction centers.

To estimate the structural and functional contribution of PSII to PI$_{ABS}$, the relationships between PI$_{ABS}$ and ET$_0$/CS$_0$, TR$_0$/CS$_0$ and ABS/CS$_0$ are presented (Figure 5E). As a result, CA-caused PI$_{ABS}$ decrease was highly related to the reduction of ABS/CS$_0$ and ET$_0$/CS$_0$, especially for ET$_0$/CS$_0$, but not TR$_0$/CS$_0$. In other words, CA-caused loss of PSII overall

![Figure 5. Analysis of the linear relationship between different selected JIP-test parameters after A. adenophora leaf discs were treated with different concentrations of cytochalasin A for 12 h. (A) The ABS/CS and TR$_0$/CS versus $F_M$. (B) The $\varphi_{Eo}$ and $R_J$ versus $V_{J}$. (C) The $R_J$ versus $\varphi_{Eo}$, $\varphi_{Eo}$ and ET$_0$/CS$_0$. (D) The $\psi_{Eo}$ and ET$_0$/CS$_0$ versus QA-reducing centers. (E) The ABS/CS$_0$, TR$_0$/CS$_0$ and ET$_0$/CS$_0$ versus PI$_{ABS}$. (F) The $\varphi_{Po}$, $\psi_{Eo}$ and QA-reducing centers versus PI$_{ABS}$. Each curve is the average of three independent experiments with around 15 repetitions.](https://example.com/figure5.png)
photosynthetic activity is mainly due to the decrease of PSII electron transport efficiency, and secondly attributed to chlorophyll concentration or the disruption of the antenna pigment assembly. This is strongly supported by the high linear correlation between PI_{ABS} and \( \psi_{Eo} \) or QA-reducing centers in the presence of different concentrations of CA. However, there is not an evident linear relationship between \( \varphi_{P_70} \) and PI_{ABS} (Figure 5F). Thus, the significant decrease of overall photosynthetic activity of PSII is really a result of antenna destruction, electron transfer interruption, and inactivation of PSII QA-reducing centers in the presence of CA.

In conclusion, CA acts in a way similar to the classical diuron-like herbicides, targeting mainly the QA site of the D1 protein and blocking PSII electron transport at the acceptor side to inhibit photosynthetic efficiency in plants.

2.5. Modeling of CA Binding to D1 Protein

In oxygenated photosynthesis in plants, algae and cyanobacteria, PSII undergoes light-driven plastoquinone reduction by electrons from water. Nevertheless, PSII herbicides interrupt this process. Numerous previous studies have shown that herbicides targeting PSII inhibit electron transfer from QA to QB by competing with native plastoquinone for its QA binding niche in the D1 protein [32,47]. The D1 protein in higher plants contains mainly five transmembrane \( \alpha \)-helices, and the QA binding site is located between the fourth and fifth transmembrane helices [32,38,48]. The amino acids that form the QA and herbicide-binding niche of D1 protein are from Phe211 to Leu275 [33].

To further verify whether CA binds to the QA site of D1 protein, CA was modeled to the QA site of \( \textit{A. adenophora} \) D1 protein based on experimental and theoretical structural information provided by Discovery Studio (version 2016, BIOVIA, San Diego, CA, USA) [49] on the binding of D1 protein by PSII herbicides (Figure 6). The molecular model of CA binding to D1 protein showed that hydrogen bonds were formed between the ethylamino hydrogen (NH) of the residue D1-His215 and the O3 oxygen atom of CA, as well as between the C18-OH hydroxyl oxygen atom of CA and CO carbonyl oxygen atom of the residue D1-Ala263 or CO carbonyl oxygen atom of D1-Ser264, respectively. Their bonding distance was 2.48 Å, 2.44 Å and 2.35 Å, respectively (Figure 6A and Table 2). The residues D1-Phe211, D1-Phe265 and D1-Phe274 also formed, respectively, a Pi hydrophobic interaction with the C16, C17=CH2 and the C16 of CA with the bound distance of 3.97 Å, 3.83 Å and 3.86 Å (Table 2). Additionally, the alkyl hydrophobic interactions between CA and the residues D1-Ile248, D1-Leu251, D1-His252 and D1-Leu271 were also probably involved in the complex stabilization of CA binding to the QA site (Table 2). Figure 6B illustrates the docked pose of CA at the QA binding site of \( \textit{A. adenophora} \) D1 protein. Hydrogen bonding with D1-Ser264 is a favorable orientation for the classical PSII herbicides atrazine and diuron [50]. Apparently, CA is not exactly the same as atrazine and diuron in the binding environment, although all target the QA site of the D1 protein.

Figure 6. The simulated modeling of cytochalasin A binding to D1 protein of \( \textit{A. adenophora} \). (A) Hydrogen bonding interactions for cytochalasin A binding to D1 protein. (B) Stereo view of cytochalasin A binding environment of D1 protein. Here, carbon atoms are shown in grey, nitrogen atoms in blue, oxygen in red and hydrogen atoms in white. The possible hydrogen bonds are indicated by dashed lines.
Table 2. Possible bonding interactions for CA binding to the D1 protein of A. adenophora.

| CA Chemical Structure | Donor          | Acceptor          | Interactions   | Bound Distance (Å) |
|----------------------|----------------|-------------------|----------------|-------------------|
| D1-Phe 211           | CAC16-CH₃      | Pi Hydrophobic    | 3.97           |
| D1-His 215 NH        | CA O3          | Hydrogen Bond     | 2.48           |
| D1-Ile 248           | CA C10-CH₃     | Alkyl Hydrophobic | 3.72           |
| D1-Leu 251           | CA C10         | Alkyl Hydrophobic | 3.77           |
| D1-His 252           | CA C10-CH₃     | Alkyl Hydrophobic | 3.81           |
| CA C18-OH            | D1-Ala 263 CO  | Hydrogen Bond     | 2.44           |
| CA C18-OH            | D1-Ser 264 CO  | Hydrogen Bond     | 2.35           |
| D1-Phe 265           | CA C17=CH₂     | Pi Hydrophobic    | 3.83           |
| D1-Leu 271           | CA C11         | Alkyl Hydrophobic | 3.91           |
| D1-Phe 274           | CA C16-CH₃     | Pi Hydrophobic    | 3.86           |

It is well known that PSII inhibitor herbicides share the same action target D1 protein, but each herbicide has its own specific orientation in binding to the Qₘₐₜ niche. The classic inhibitors of PSII can be classified as the urea/triazine, and phenolic inhibitors. Extensive studies with herbicide-tolerant mutants and crystal structure-based molecular modeling have shown that hydrogen bonding with D1-Ser264 is the key interaction for urea/triazine herbicides to bind D1 proteins, whereas the favorable orientation of phenolic herbicides to bind D1 proteins is through hydrogen bonding with His215 [32,48,51]. Here, D1-His215, D1-Ser264 and D1-Ala263 are thought to be involved in the binding pocket of CA and provide hydrogen bonds for CA (Figure 6B). As compared with the two types of PSII herbicides, the binding environment of CA in D1 protein overlaps but differs from theirs, and residue D1-Ala263 is a novel site of action. Overall, the binding orientation of CA to D1 protein appears to be more complex than that of classical herbicides to D1 protein.

Some natural products with herbicidal properties also exhibit complex binding orientations to the Qₘₐₜ site. According to the constructed molecular model of patulin (PAT) [38], binding to D1 protein, D1-His252 provides a major hydrogen bond to the O₂ carbon-based oxygen atom of PAT. This mode of binding to D1 proteins is not consistent with either urea/triazine family inhibitors or phenolic inhibitors and may be the result of differences in the characteristic groups. The molecular interaction model of TeA [50] and Arabidopsis D1 protein indicates that the residue Gly256 of D1 protein plays a key role in TeA binding to the Qₘₜ niche of D1 protein. Apparently, this binding behavior is also different from that of classical herbicides. In this study, the molecular interaction model of CA binding to the Qₘₜ site indicated that residues D1-His215, D1-Ala263 and D1-Ser264 were involved in the formation of hydrogen bonds, respectively, promoting the binding of CA to D1 protein. Compared with the classical PSII herbicides and natural PSII inhibitors mentioned above, CA has a unique binding orientation for residue D1-Ala263 at the Qₘₜ site. Therefore, CA is a novel natural photosynthetic inhibitor with potential for future development as a bioherbicide. Further verification of the exact binding environment of CA, however, is required by crystallographic data and mutant experiments.

3. Materials and Methods

3.1. Plants and Chemicals

Invasive weed A. adenophora was germinated on a mixture of perlite-vermiculite–peat (0.5:1:3, v/v) at 20–25 °C under 200 μmol (photons) m⁻² s⁻¹ white light (day/night, 12 h/12 h) and 70% relative humidity in the greenhouse. After 180 days of growth, the leaves of plants were sampled.

C. reinhardtii wild-type strain was obtained from the Freshwater Algae Culture Collection at the Institute of Hydrobiology (FACHB-col-lection, Chinese Academy of Science, China). Cells were grown in liquid Tris-acetate-phosphate (TAP) medium at 25 °C under white light (100 μmol (photons) m⁻² s⁻¹) with a 12 h photoperiod and shaken once every 12 h. The total biomass was determined by optical density of cell suspensions in a spectrophotometer (UV-1800, Shimadzu, Kyoto, Japan) at 750 nm (A₇₅₀). The cells of
the logarithmic phase (approximately 3 or 4 days, \(A_{750}\) of about 0.65) were collected and washed twice with distilled water, then resuspended with buffer A. The buffer contained 20 mM HEPES-KOH (pH 7.5), 350 mM sucrose and 2.0 mM MgCl\(_2\). The cells were used for the subsequent experiments.

CA (CAS No. 14110-64-6) was purchased from Sigma-Aldrich (Shanghai, China), and other common chemical reagents used in this work were purchased from Aladdin (Shanghai, China). CA was prepared in 100% acetone and diluted in distilled water as required. The final concentration of acetone in each experiment did not exceed 10% (v/v).

### 3.2. Phytotoxicity Assessment

For the normal images of \textit{A. adenophora}, intact detached-leaves from healthy plants of \textit{A. adenophora} were washed with distilled water, dried with sterilized-filter papers, and transferred onto wet sterilized-filter papers in Petri dishes. Leaves were slightly punctured on their abaxial margin with a needle. The 20 \(\mu\)L droplet of 0 (mock, 10% acetone), 100, 200, 400 and 800 \(\mu\)M CA solutions were added onto the wound site of leaves. All samples were maintained at 25 \(^\circ\)C for 12 h in a growth chamber, under approximate 200 \(\mu\)mol (photons) m\(^{-2}\) s\(^{-1}\) white light (day/night, 12 h/12 h). Results were recorded with a Canon G15 camera (Canon, Tokyo, Japan) and diameters of leaf necrotic lesions were determined with a vernier caliper (ROHS HORM 2002/95/EC, Xifeng, Qingyang, China).

### 3.3. Measurement of PSII Oxygen-Evolution Rate of \textit{C. reinhardtii}

A Clark-type oxygen electrode (Hansatech Instruments Ltd., King’s Lynn, UK) was used to determine the oxygen evolution rate of \textit{C. reinhardtii} according to [39]. Before measurements, CA was added to 2 mL cell suspensions (\(A_{750}\) of 0.65) to make final concentrations as indicated (0, 12.5, 25, 50 and 100 \(\mu\)M), and then the cells were incubated for 3 h in darkness at 25 \(^\circ\)C. Treated cells containing 45 \(\mu\)g chlorophylls were added into the PSII reaction medium of 4.0 mL, which included 50 mM HEPES-KOH buffer (pH 7.6), 4 mM K\(_3\)Fe(CN)\(_6\), 5 mM NH\(_4\)Cl and 1 mM p-phenylenediamine. After the reaction mixture was illuminated with 400 \(\mu\)mol m\(^{-2}\) s\(^{-1}\) red light for 1 min, the oxygen evolution rate was recorded. The independent experiment was repeated three times.

### 3.4. Chl \(a\) Fluorescence Imaging

A MAXI-version of the pulse-modulated Imaging-PAM M-series fluorometer (Heinz Walz GmbH, Effeltrich, Germany) was used to determine the Chl \(a\) fluorescence imaging [44]. Before measurements, samples were adapted for 0.5 h in the dark under the imaging system camera after focusing the camera. Under 0.25 \(\mu\)mol (photons) m\(^{-2}\) s\(^{-1}\) measuring light, 110 \(\mu\)mol (photons) m\(^{-2}\) s\(^{-1}\) actinic light and 6000 \(\mu\)mol (photons) m\(^{-2}\) s\(^{-1}\) saturation pulse light, fluorescence images were monitored. The following parameters were measured directly: \(F_V/F_M\) and ETR.

For the measurement of \textit{C. reinhardtii} cells, 200 \(\mu\)L of suspensions (\(A_{750}\) of about 0.65) with 10% acetone (mock) and CA were added into the 96-well black microtiter plate to a final CA concentration of 20, 30 and 40 \(\mu\)M, respectively. The treated cells were incubated for 2.5 h at 25 \(^\circ\)C under 100 \(\mu\)mol (photons) m\(^{-2}\) s\(^{-1}\) white light, and then samples were adapted in darkness for 0.5 h before fluorescence determination.

### 3.5. Chl \(a\) Fluorescence Kinetics OJIP Curves and JIP-Test

A plant efficiency analyzer (Handy PEA, Hansatech Instruments Ltd., King’s Lynn, Norfolk, UK) was used to measure the Chl \(a\) fluorescence kinetics OJIP curves. For the measurement, 7-mm diameter leaf discs of \textit{A. adenophora} were washed with distilled water and incubated for 12 h in CA solutions with 0 (10% acetone, mock), 25, 50, 100 and 200 \(\mu\)M concentrations, respectively. Meanwhile, to verify the time-dependent effect of CA, leaf discs were also treated with 50 \(\mu\)M CA for 0, 3, 6 and 12 h, respectively. Samples were well adapted to darkness for 0.5 h prior to measurement. Raw fluorescence transients
OJIP was transferred to a spreadsheet by Handy PEA V1.30 and BiolyzerHP3 software. The independent experiment was repeated three times with around 15 repetitions. The raw data of the fluorescence kinetics OJIP curves were analyzed using the JIP-test [41,52]. Detailed JIP-test parameters containing formulas, equations and definitions are listed in Table 1, which are according to Strasser et al. [41] and Chen et al. [45].

3.6. Modeling of CA in D1 Protein of A. adenophora

The target D1 protein of A. adenophora can be provided on the basis of the amino acid sequence (reference sequence no. YP 004564352.1). The sequence of amino acid was obtained from NCBI, in FASTA format. Evolutionary related protein structures were searched for by the BLAST databases through the SWISS-MODEL Template Library (SMTL) [53]. Searched templates of D1 protein were estimated using Global Model Quality Estimate (GMQE) and Quaternary Structure Quality Estimate (QSQE) and ranked by the expected quality of the resulting models. The protein structures of top-ranked templates were selected from the Protein Data Bank (PDB) and built the homology model of A. adenophora D1 protein by the Protein Module of Discovery Studio. The chemical structure of CA was constructed with the software of ChemBioDraw Ultra 14.0 (CambridgeSoft, Cambridge, MA, USA), and the Chem3D Pro 14.0 (CambridgeSoft, Cambridge, MA, USA) was used to minimize energy. The docking was performed by DS-CDocker in Discovery Studio 2016 (BIOVIA, San Diego, CA, USA). The polar was added to the protein during energy minimization and molecular refinement.

3.7. Statistical Analysis

One-way ANOVA was carried out and means were separated by DUNCAN LSD at 95% using SPSS Statistics 20.0.

4. Conclusions

CA is a natural mycobacterial metabolite that is widely known for its targeting of actin microfilaments to produce a series of interesting physiological phenomena. Our study reveals some interesting effects of CA on plant PSII and finds that CA has potential herbicidal activity. Based on the analysis of Chl α fluorescence rise kinetics and PSII oxygen evolution rate, CA inhibits photosynthesis by occupying the Q$_B$ site in the D1 protein, thus blocking the electron transfer from Q$_A$ to Q$_B$. The loss of overall photosynthetic activity of PSII is mainly due to the reduction of electron transfer efficiency, the destruction of antenna pigments and the inactivation of PSII reaction centers in the presence of CA. This mode of action is similar to that of the classical PSII herbicide diuron. The molecular docking model of CA binding to A. adenophora D1 protein further suggests that the direct target of CA is the Q$_B$ site of D1 protein in PSII. It is proposed that CA forms potential hydrogen bonds with residues D1-His215, D1-Ala263 and D1-Ser264, respectively. Among them, D1-Ala263 is a novel site of action of CA different from the urea/triazine and the phenolic inhibitors. The inhibitory effect of CA on PSII allows CA to be used directly as a PSII inhibitor in various studies in the future. Considering the high development costs of chemical herbicides and the prevalence of resistant weeds, the unique binding of CA to D1 protein provides a new idea for the design of novel and efficient herbicide molecules to effectively manage weeds.

**Author Contributions:** S.C. designed the experiments. M.J., Q.Y., H.W., X.W. and Y.G. carried out experiments. S.C., Y.G., H.W., M.J., R.J.S., S.Q. and X.W. analyzed the data. M.J., H.W., Y.G., J.S. and Z.L. wrote the paper. S.C. revised the manuscript and prepared the final version. All authors have read and agreed to the published version of the manuscript.

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