Cu and Zn Stress affect the photosynthetic and antioxidative systems of alfalfa (Medicago sativa)

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ABSTRACT
Cu and Zn are common and potentially harmful heavy metals to plants, animals and humans. Herein, we investigated the effects of Cu and Zn stress on the photosynthesis and tolerance mechanism of alfalfa plants to ROS using fluorescence and biochemical methods. The results showed that Cu stress significantly reduced the chlorophyll content of the leaves, while Zn stress only reduced the Chl a content. The Fv/Fm decreased significantly under Cu stress but was not affected by Zn treatment. However, the Pmax of the leaves were sensitive to Cu and Zn stress. Both Cu and Zn stress resulted in the weakening of the ability of PQ library to accept electrons, the damage of OEC, and the inhibition of the electron transfer from QA to QB. Moreover, Cu stress also dissociated the thylakoids of leaves, but Zn stress did not significantly damage it. In Cu and Zn stressed leaves, the reduction of RC/CSm significantly increased the ABS/RC and TR/RC values. When the stress intensified, the value of DI/RC increased indicated a plant self-protection mechanism that eliminates excess energy in the PSI reaction center and increases the energy for heat dissipation per unit reaction center. Cu stress significantly increased the O2 production rate, H2O2 content, and MDA accumulation in the leaves. However, Zn stress exhibited a minimal effect on the ROS production and oxidative damage in the alfalfa leaves but increased the O2 production rate at the concentration of 800 μmol·L⁻¹. Cu stress increased the activities of SOD, POD, CAT, APX, and GPX in the leaves; however, leaves adapts to Zn stress by enhancing the activities of SOD and GPX. Thus under Cu stress, the degree of photoinhibition and oxidative damage in alfalfa leaves were significantly higher than under Zn stress.

1. Introduction
Heavy metals are elements whose density exceeds 4.5 kg/dm³, including chromium (Cr), mercury (Hg), lead (Pb), copper (Cu), zinc (Zn), cadmium (Cd), arsenic (As), etc (Kavamura and Esposito 2010). The common heavy metal pollutants worldwide are Cr³⁺, Cd²⁺, Hg²⁺, As³⁺, Cu²⁺, and Zn²⁺ (Su et al. 2014; Wjcik et al. 2015), mainly resulting from rock weathering and human activities. With the rapid development of mining, metallurgy, textile, and other manufacturing industries, several heavy metals are being discharged into the atmosphere, water sources, and soil (Facchinelli et al. 2001; Li 2019), resulting in water pollution (Montalvo et al. 2014), air pollution (Popov et al. 2014) and soil pollution (Manu et al. 2016). The misuse of drugs and fertilizers in modern agriculture has also contributed to soil pollution with heavy metals (Zhang et al. 2020). These soil-polluting heavy metals can be enriched by plants, eventually causing damage to plants (Schwalbert et al. 2021). Heavy metals damage plants in multiple steps; they destroy the chlorophyll making the plants lose the green leaf color, which subsequently affects photosynthesis and leads to malnutrition and even death of plants (Liu and Hallenbeck 2016). Moreover, heavy metal ions can capture the channel position of essential element ions in plants, disrupting the normal physiological and biochemical processes. Heavy metal ions can also destroy plant cell DNA, cellular membrane structures, electron transport chain, and other physiological activities, causing irreversible damage to plants (Chaabene et al. 2018; He et al. 2021).

Generally, plants can metabolize reactive oxygen species (ROS) and maintain a dynamic balance (Baxter et al. 2014). However, when plants are severely stressed with heavy metals, the ROS accumulate in large quantities, damaging various physiological functions, such as enzyme activities, photosynthetic pigments synthesis, and accelerating the photosynthetic pigments degradation (Martin and Sies 2017). The ROS also attacks the plant biofilm system and induces peroxidation of the unsaturated fatty acids, leading to the destruction of the membrane structure. This increases the non-selective permeability of the cell membrane, extravasation of cellular ions, and disruption of the cellular metabolic process (Scandalias 2002). Thus, plants activate the
antioxidant system when the ROS exceeds a certain limit to protect their cells and tissues from the oxidative damage of ROS. The antioxidative defense system is a precise and efficient ROS scavenging system composed of various antioxidant substances, including enzymes such as SOD, CAT, POD, and APX (Miller et al. 2008). Several studies have shown that plant adaptability and tolerance to heavy metals are closely associated with the plant antioxidative system, and strong antioxidant capacity can improve plant adaptability (Halliwell and Gutteridge 1985; Ali et al. 2014; Baxter et al. 2014). For example, Cu, an essential trace element and a cofactor of many enzymes such as ascorbic acid oxidase and SOD (Fan et al. 2011), induces a high accumulation of ROS. This significantly increases the malondialdehyde content, damaging the leaf cells (Deng et al. 2013; Leitao et al. 2021), but improves the activities of antioxidative enzymes such as SOD, GPX, APX, and CAT (Buapet et al. 2018; Gong et al. 2019). Additionally, Zn, a trace metal necessary for the normal growth and development of plants (Broadley et al. 2007), can also cause poisoning and affect the normal ROS balance in plants. It has been reported that Zn stress increases POD activity but inhibits CAT activity in pigeon pea (Cajanus cajan (L.) Millspaugh) leaves (Madhava Rao 2000). Zn stress also inhibits APX activity in kidney bean (Phaseolus vulgaris) (Ann et al. 2001); however, some studies have reported that it enhances APX activity in mustard (Brassica juncea) (Prasad et al. 1999) and pigeon pea (Cajanus cajan (L.) Millspaugh) (Madhava Rao 2000) leaves.

Alfalfa (Medicago sativa L.) is a perennial leguminous forage widely cultivated because of its high yield and good quality and strong tolerance to abiotic stresses such as drought (Huang et al. 2018), low temperatures (Xu et al. 2020), and saline-alkali (Zhang et al. 2018). However, there are few studies on the photosynthetic function and antioxidant mechanisms of alfalfa under Cu and Zn stress. In-depth studies on the antioxidant mechanisms of plants under different heavy metal stress can provide basic theoretical data for improving plant resistance to heavy metal stress. Therefore, this study evaluated the chlorophyll fluorescence parameters and the activities of enzymes related to ROS production and scavenging in Cu- and Zn-stressed alfalfa leaves to reveal the toxic and adaptive mechanisms of alfalfa under heavy metal stress through photosynthesis, ROS metabolism, and antioxidant mechanism.

1. Materials and methods

1.1. Experimental materials and treatments

We selected mature and plump seeds of Medicago sativa cv. Zhaodong, with relatively consistent sizes, for germination in culture dishes. After the embryos grew to about 0.5 cm, we selected the germinating seeds with relatively similar growth rates and sowed them in a 1:1:1 mixture of peat soil, perlite and vermiculite. Each seedling was planted per bowl by covering the seed surface about 1 cm with soil, followed by culturing at room temperature under 400 μmol·m⁻²·s⁻¹ of light intensity and 12/12 h photoperiod (light/dark) using LED lamps. The seedlings were carefully removed from the culture medium when their height reached approximately 20 cm, and the culture medium attached to the root surface was washed off. Thereafter, the seedlings were fixed on a black foam board with a sponge and incubated in water-tight culture boxes (width of 25 cm and a height of 30 cm) containing a half-strength Hoagland nutrient solution. Each water culture box (hydroponic incubator) contained 10 L of the nutrient solution and was continuously aerated using electric air pumps. Ten seedlings were cultured in each hydroponic incubator, and the nutrient solution was changed every 3 days. After 30 days of the seedlings culturing, CuSO₄ and ZnSO₄ were added to the nutrient solution to the final Cu²⁺ and Zn²⁺ concentrations of 100, 200, 400, and 800 μmol·L⁻¹, each. The half-strength Hoagland nutrient solution without Cu²⁺ and Zn²⁺ was used as the control (represented by CK). The photosynthetic parameters and physiological indexes of the alfalfa leaves were measured on the 7th-day post-treatment.

1.2. Determination of photosynthetic parameters and physiological indexes

Determination of chlorophyll content: Fresh leaves (about 0.5 g) without main vein from each group were immersed in 2 mL of the mixture of acetone and ethanol (V:V = 1:1) and oscillated in the dark until the green completely faded. Absorbance values at 665 and 649 nm were measured using a spectrophotometer (Agilent Technologies, China). The content of chlorophyll a (Chl a), chlorophyll b (Chl b), total chlorophyll (Chl a+b), and chlorophyll a/b (Chl a/b) were measured via spectrophotometry (Pirie and Mullins 1976).

Physiological indicators related to the ROS metabolism: Thiobarbituric acid colorimetry was used for MDA content determination (Ernst and Nordenbrand 1977), superoxide anion (O₂⁻) production rate was evaluated using the method by Elstner and Heupel (1976). Hydrogen peroxide (H₂O₂) was extracted using 5% (w/v) trichloroacetic acid and measured according to the method by Patterson et al. (1984). Conversely, SOD, POD, and CAT activities were determined using the method by Wang and Huang (2015). The activity (1U) of SOD is defined as the amount of enzymes required to reduce NBT to half of that of the control group. The activity (1U) of CAT is defined as the reduction of an absorbance at 240 nm (A₂₄₀) by 0.1, and the activity (1U) of POD is defined as the reduction of an absorbance at 470 nm (A₄₇₀) by 0.01. APX and GPX activities were measured using their respective assay kits (Suzhou Comin Biotechnology Co., Ltd).

Evaluation of the OJIP curve: The fully expanded and functional alfalfa leaves were selected from each treatment, and their dark adaptation was measured for 30 min using a dark adaptation clip. Subsequently, the OJIP curves of the leaves were measured, five times each, by an Hansatech multifunctional plant efficiency instrument (M-PEA), and the OJIP curves were analyzed using a JIP-test. A method by Strasser (1997) was then used to calculate the following parameters: 1) maximum photochemical efficiency of PSII (Fₚ/Fₘ), 2) photosynthetic performance indexes based on absorbed light energy (Pᵣₜₐₜ), 3) absorbed light energy per unit reaction center (ABS/RC), 4) electron transfer energy per unit reaction center (ETᵣₒ/RC), 5) dissipated energy per unit reaction center (DIᵣₒ/RC), 6) absorbed energy per unit area (ABS/CSₙ), 7) electron transfer energy per unit area (ETᵣₒ/CSₙ), 8) heat-dissipated energy per unit area (DIᵣₒ/CSₙ), and 9) the number of active reaction centers per unit area (RC/CSₙ).
The OJIP curves were further normalized to O-P, O-J, and O-K using the formulae $V_{O-P} = (F_t - F_o)/(F_m - F_o)$, $V_{O-J} = (F_t - F_o)/(F_t - F_{o,J})$, and $V_{O-K} = (F_t - F_o)/(F_t - F_{o,K})$, respectively, generating the $V_{O-P}$, $V_{O-J}$, and $V_{O-K}$ curves, respectively. The $F_t$ and $F_o$ represented the relative fluorescence intensities at 2.0 and 0.3 ms on the OJIP curves, respectively. The $F_t$ represented the relative fluorescence intensity at each time point on the OJIP curves. Moreover, the relative variable $V_{O,P}$ represented the relative $a + b$ contents decreased significantly (Figure 2-A, 2-B, 2-C). The decrease in Chl a was greater than in Chl b; therefore, higher Cu concentrations also reduced the Chl a/b content of alfalfa leaves (Figure 2-D). Similar to its phenotypic effects, Zn treatment had significantly reduced effects on chlorophyll content of alfalfa leaves compared to Cu. It significantly decreased the Chl a, Chl a + b, and Chl a/b contents of the treated plants, only at a concentration of 800 μmol L$^{-1}$ (Figure 2-A, 2-C), but had no significant effect on Chl b content compared to CK plants (Figure 2-B, 2-D).

### 2. Results

#### 2.1. Plant phenotype

The 100 and 200 μmol L$^{-1}$ concentrations of Cu treatment had little effect on the alfalfa phenotype, as shown in Figure 1. Upon increasing the concentration to 400 and 800 μmol L$^{-1}$, the alfalfa leaves turned yellow, while a concentration of 800 μmol L$^{-1}$ resulted in darkening and abscission of alfalfa roots leaves, respectively. Meanwhile, the different concentrations of Zn treatment had significantly lesser effects on the alfalfa phenotype than Cu. Although alfalfa roots were also darkened by a Zn concentration of 800μmol L$^{-1}$, the leaves did not show pronounced chlorosis.

#### 2.2. Chlorophyll content

The Cu concentrations of 100 and 200 μmol L$^{-1}$ Cu had no significant effects on Chl a, Chl b, Chl a + b, and Chl a/b contents of alfalfa leaves; however, when the concentrations increased to 400 and 800 μmol L$^{-1}$, Chl a, Chl b, and Chl a + b contents decreased significantly (Figure 2-A, 2-B, 2-C). The decrease in Chl a was greater than in Chl b; therefore, the concentrations of Cu concentration reached 200 μmol L$^{-1}$.

#### 2.3. OJIP curve and photochemical activity of PSII

The relative fluorescence intensities ($F_o$ and $F_m$) points O and P of the OJIP curve of the alfalfa leaves did not change significantly. However, the relative fluorescence intensity ($F_t$) of the J point showed a significant increase compared with CK, at Cu concentrations of 100 and 200 μmol L$^{-1}$. The $F_t$ and $F_o$ of the treated plants increased significantly compared with CK when Cu concentration increased to 400 and 800 μmol L$^{-1}$. Conversely, 800 μmol L$^{-1}$ of Cu also significantly reduced the $F_m$ of alfalfa leaves (Figure 3-A). There were no significant differences in the $F_o$ of the OJIP curve compared with CK; however, the $F_m$ was slightly higher than that of the CK at different Zn concentrations, while $F_t$ increased greatly with Zn concentration (Figure 3-B). The $F_t/F_m$ did not significantly change, but $PI_{ABS}$ significantly decreased compared to CK, at Cu concentrations of 100 and 200 μmol L$^{-1}$, as shown in Figure 3-C and 3-D. Additionally, the $F_t/F_m$ decreased by 10.59% ($P < 0.05$) and 19.64% ($P < 0.05$) at the Cu concentrations of 400 and 800 μmol L$^{-1}$, respectively, while $PI_{ABS}$ reduced by 92.57% ($P < 0.05$) and 90.44% ($P < 0.05$), the same Cu concentrations, respectively. There were no significant differences in $F_t/F_m$ among the different Zn concentrations, but $PI_{ABS}$ was significantly lower than the CK when Zn concentration reached 200 μmol L$^{-1}$.

#### 2.4. Normalized OJIP curves and their relative variable fluorescence at the characteristic points

Under different Cu and Zn concentrations, the relative variable fluorescence of $V_I$ at 2 ms on standardized O-P curve (Figure 4-A, 4-B), $V_K$ at 0.3 ms on standardized O-J curve (Figure 4-C, 4-D), and $V_L$ at 0.15 ms on standardized O-K curve (Figure 4-E, 4-F) were significantly different compared to CK. Moreover, the relative variable fluorescence of $V_K$, $V_I$, and $V_L$ were significantly greater in Cu than in Zn treatment at each characteristic point.

Compared with CK, the $V_K$, $V_I$, and $V_L$ of the alfalfa leaves increased significantly with the different Cu concentrations,
as shown in Figure 4-G, 4-H, and 4-I. Meanwhile, the $V_I$ increased significantly under different concentrations of Zn, but $V_I$ increased significantly only at the Zn concentration of 800 μmol·L$^{-1}$, compared to CK. Although the $V_K$ increased to varying degrees under different concentrations of Zn compared to CK, the difference was significant only at Zn concentrations of 400 and 800 μmol·L$^{-1}$ (Figure 4-I). The $V_I$ increased significantly at Cu concentrations of 400 and 800 μmol·L$^{-1}$ but had no significant changes at different concentrations of Zn treatment (Figure 4-J).
Figure 4. Effects of Cu and Zn treatment on standardized O-P curve (A, B), standardized O-J curve (C, D), standardized O-K curve (E, F), V_J (G), V_I (H), V_K (I) and V_L (J) in alfalfa leaves.

Note: The data in the figure are from three biological repeats (n = 3), and represent means ± standard error (SE). Significant differences were expressed by different letters (p< 0.05).
2.5. PSII per unit reaction center and the energy distribution parameters and number of active reaction centers per unit area

The ABS/RC, TRo/RC, DRo/RC, and DIO/CSm of the alfalfa leaves increased with the increase of Cu concentration; however, there were no significant differences among parameters at concentrations 100 and 200 μmol·L⁻¹ of the Cu treatment compared with CK. However, the parameters increased significantly compared with CK at Cu concentrations of 400 and 800 μmol·L⁻¹. The different concentrations of Cu treatment did not change the ETo/RC and TRo/CSm of the alfalfa leaves, but ETo/CSm was significantly reduced compared to CK at Cu concentrations of 400 and 800 μmol·L⁻¹ (Figure 5-A). The range of energy allocation parameters of the alfalfa leaves exhibited minimal changes at different concentrations of Zn treatment, as shown in Figure 5-B. The other parameters did not change significantly except for the significant increase in ABS/RC, TRo/RC, DRo/RC, and DIO/CSm at different Zn concentrations.

Additionally, the RC/CSm decreased by 54.18% (P < 0.05) and 58.01% (P < 0.05) at Cu concentrations of 400 and 800 μmol·L⁻¹, respectively, compared with CK (Figure 5-C). The different concentrations of Zn also reduced RC/CSm to varying degrees; however, differences were not significant, and the extent of reduction was significantly lower than that of Cu treatment (Figure 5-C).

2.6. Physiological analysis

The generation rate and contents of O₂⁻, H₂O₂, and MDA in alfalfa leaves did not change significantly compared with CK at 100 and 200 μmol·L⁻¹ of Cu treatment but increased significantly when Cu concentration increased to 400 and 800 μmol·L⁻¹. Conversely, except for the increase in the generation rate of O₂⁻ at 800 μmol·L⁻¹, the other parameters did not change significantly at different concentrations of Zn treatment (Figure 6-A, 6-B, 6-C). The activities of SOD, POD and GPX in the alfalfa leaves increased with the increase of Cu concentration, had no significant differences compared to CK at 100 and 200 μmol·L⁻¹ of Cu treatment (Figure 6-D, 6-E, 6-H). Moreover, the CAT and APX activities showed no significant differences in compared with CK at 100 and 200 μmol·L⁻¹ of Cu treatment but decreased significantly when Cu concentrations increased to 400 and 800 μmol·L⁻¹ (Figure 6-F, 6-G). There were minimal changes in the activities of SOD, POD, CAT, APX, and GPX in alfalfa leaves at different Zn concentrations, and at 800 μmol·L⁻¹ of Zn concentration, the SOD and GPX activities were significantly higher than in CK (Figure 6-D, 6-H).

3. Discussion

Chlorophyll is an important component of photosynthesis that reflects the photosynthetic strength indexes of plants. Its higher contents in plants are essential for maintaining normal photosynthesis processes under stressful conditions (Zhang et al. 2020). The decrease in chlorophyll content has been reported to inhibit the capture and utilization of light energy (Zhang et al. 2016). In this study, the Cu concentration of 400 μmol·L⁻¹ significantly decreased the chlorophyll contents of the alfalfa leaves. Similarly, the Zn concentration at 100 and 200 μmol·L⁻¹ of Cu treatment but increased significantly when Cu concentration increased to 400 and 800 μmol·L⁻¹. Conversely, except for the increase in the generation rate of O₂⁻ at 800 μmol·L⁻¹, the other parameters did not change significantly at different concentrations of Zn treatment (Figure 6-A, 6-B, 6-C). The activities of SOD, POD and GPX in the alfalfa leaves increased with the increase of Cu concentration, had no significant differences compared to CK at 100 and 200 μmol·L⁻¹ of Cu treatment (Figure 6-D, 6-E, 6-H). Moreover, the CAT and APX activities showed no significant differences in compared with CK at 100 and 200 μmol·L⁻¹ of Cu treatment but decreased significantly when Cu concentrations increased to 400 and 800 μmol·L⁻¹ (Figure 6-F, 6-G). There were minimal changes in the activities of SOD, POD, CAT, APX, and GPX in alfalfa leaves at different Zn concentrations, and at 800 μmol·L⁻¹ of Zn concentration, the SOD and GPX activities were significantly higher than in CK (Figure 6-D, 6-H).
concentration of 800 μmol·L\(^{-1}\) significantly decreased the Chl a content but did not significantly change the Chl b content. This suggests that the alfalfa leaves are more sensitive to Cu than Zn stress and that Chl a is more sensitive to Zn stress than Chl b. Excessive Cu\(^{2+}\) and Zn\(^{2+}\) enter chloroplasts and replace Mg\(^{2+}\) in the chlorophyll molecules, disrupting photosynthesis (Kupper et al. 1998; Ambrosini et al. 2018; Schwalbert et al. 2019). It is reported that low concentrations of Cu\(^{2+}\) (<300 mg/kg) increase the chlorophyll contents of ‘Hanfu’ apple seedlings, thus keeping their leaves healthy. However, the Cu\(^{2+}\) concentration exceeding 300 mg/kg stresses the plants and reduces their chlorophyll content and photosynthetic rate (Bu 2019). When Chen et al. (2015) applied 600 mg/kg of Cu\(^{2+}\) to Phyllostachys edulis (moso), the photosynthetic efficiency index of Chl a/b decreased significantly in its leaves, indicating that high Cu concentrations inhibits chlorophyll synthesis and reduces the photosynthetic efficiency of the plant. Consistently, Sagardoy et al. (2010) found that excessive Zn reduces the content of photosynthetic pigments such as Chl a and Chl b and affects the electron transfer of PSII in sugar beet (Beta vulgaris L.). The inhibition of chlorophyll synthesis turns the leaves yellow and destroys the photosynthesis of plants, inhibiting their normal growth, development, and metabolism (Bruné et al. 2010).

Chlorophyll fluorescence kinetics is considered a fast and non-invasive technique for studying plant photosynthetic functions. It plays a unique role in measuring the absorption, transmission, dissipation, and distribution of light energy during the photosynthesis process (Govindjee and Papaerougi 2004; Baker 2008). Since photosynthesis inhibition first affects the PSII, the response mechanism of PSII to the stress is considered the most important survival strategy by which plants adapt to photosynthetic stress. Moreover, the decline of photosynthetic rate affects the absorption, transmission, and transformation of light energy, and the photochemical activity in plants (Zhang et al. 2013). The photochemical efficiency of PSII (F\(_{v}/F_{m}\)) and the photosynthetic
performance index ($P_{ABS}$) are important parameters for studying the photosynthetic physiological state of plants (Maxwell and Johnson 2000). In this experiment, the $F_v/F_m$ significantly decreased when the concentration of Cu reached 400 μmol·L$^{-1}$. Similarly, the 100 μmol·L$^{-1}$ of Cu and 200 μmol·L$^{-1}$ of Zn treatments also significantly reduced the $P_{ABS}$. These results show that Cu and Zn stress damaged the photosynthetic performance of the alfalfa leaves. Tuba et al. (2010) and Baycu et al. (2016) also demonstrated that heavy metal stress inhibits the photosynthetic electron transfer rate and PSI activity, thus hindering photosynthesis.

Heavy metals have multiple action points for damaging the electron transport chain (Vasi et al. 2007; Zhang et al. 2020); therefore, we specifically analyzed the relative variable fluorescence changes at points L, K, J, and I after standardizing the original OJIP curves according to O-P. The $V_L$ of the leaves treated with 200, 400 and 800 μmol·L$^{-1}$ of Cu, and 800 μmol·L$^{-1}$ of Zn increased significantly compared with CK, indicating that the electron transfer from $Q_A$ to $Q_B$ was inhibited after its transfer from Pheo to $Q_A$ (Strasser 1997; Haldimann and Strasser 1999). Consequently, it is postulated that the change in $V_L$ reflects the heterogeneity of the PQ library during the electron transfer process from $Q_A$ to $Q_B$ (Govindje 1995; Li et al. 2005). We demonstrated that 100 μmol·L$^{-1}$ of Cu or Zn significantly increased the $V_L$ value, indicating that the electron acceptance of PQ is sensitive to Cu and Zn metal stress. The $V_K$ value of the leaves increased at 100, 200, 400, and 800 μmol·L$^{-1}$ of Cu, and 800 μmol·L$^{-1}$ Zn treatments, suggesting damage of the oxygen releasing complex (OEC). The OEC damage limits electron transfer from the electron donor side to the secondary electron donor $Y_{Zn}$, resulting in an electron imbalance between the donor and the reaction center and between the reaction center and the receptor side (Strasser 1997; Zhang et al. 2012; Salau et al. 2015). Moreover, the increase in the relative variable fluorescence ($V_L$) at the I point of the OJIP curve indicates thylakoid dissociation (Esselmine et al. 2012). The thylakoid membrane contains photosynthetic pigments and electron transport chain components where the light reaction occurs; therefore, thylakoids dissociation disrupts the function of the leaf photosystem, thus reducing photosynthesis. In this experiment, leaf thylakoids were damaged at 400 and 800 μmol·L$^{-1}$ of Cu treatment but were not affected by Zn stress. Muzammal et al. (2019) also reported that high concentrations of Cu destroy the structure and function of thylakoids and eventually inhibit photosynthesis.

Changes in the specific activity parameters of the unit reaction center determine the absorption and utilization of light energy and the reaction center activity (Strasser et al. 2008). Normally, the PSII reaction center captures light energy for the next-stage energy transfer, and the remaining energy undergoes heat dissipation (Wang et al. 2019). Our results show that $ABS/RC$ and $TR_/RC$ increased significantly in the leaves treated with 400 and 800 μmol·L$^{-1}$ of Cu and 100 μmol·L$^{-1}$ of Zn. This is because reducing the number of active reaction centers per unit area after Cu and Zn stress increases the function of the remaining active reaction centers, enhancing specific activity parameters per unit reaction center. A phenomenon also supported by the decrease in $RC/CS_m$ values. Furthermore, the increase in $DI_/RC$ value when the concentration of Cu and Zn reached 400 μmol·L$^{-1}$ indicated a plant self-protection mechanism that reduces excess energy in the PSII reaction center and increases the energy for heat dissipation per unit reaction center.

Excess electron leakage accumulates in the photosynthetic electron transport chain when the photosynthesis is inhibited and convert the free cellular O$_2$ to O$_2$, which is then reduced to H$_2$O$_2$ by SOD. Thereafter, the inhibition of the photosynthetic oxygen releases the OEC complex, which catalyzes the incomplete cleavage of water to produce H$_2$O$_2$ (Noctor et al. 2017; Foyer 2018). The accumulation of ROS, such as O$_2$ and H$_2$O$_2$, lead to the peroxidation of the plant cell membrane to produce malondialdehyde (MDA), aggravating membrane lipid peroxidation and reducing the integrity of the plant membrane system (Deng et al. 2013; Zhang et al. 2020; Yang et al. 2021). At 400 and 800 μmol·L$^{-1}$ of Cu treatment, we observed a significant increase in the O$_2$ production rate, H$_2$O$_2$ content, and MDA accumulation; however, Zn treatment had fewer effects on ROS production and oxidation in alfalfa leaves. The O$_2$ production rate increased, while H$_2$O$_2$ and MDA contents had no significant changes at the Zn concentration of 800 μmol·L$^{-1}$. Excessive cellular O$_2$ is eliminated by SOD scavenging (Lee et al. 2021), which was enhanced by the excessive Cu and Zn in our experiment because Cu and Zn are essential metal auxiliary groups of the Cu/Zn-SOD that maintain the SOD functions (Wang et al. 2004). Boojar MMA (2007) and Pandey et al. (2002) also reported that SOD activity increases significantly in plant leaves under high concentrations of Cu$^{2+}$ and Zn$^{2+}$. Hammerschmitt et al. (2020) also found that SOD activity was usually increased with high Cu and Zn concentrations. Furthermore, O$_2$ can also be removed by POD and CAT reduced to H$_2$O$_2$ by SOD (Mi and Shin 2003). In this study, POD and CAT activities increased in alfalfa leaves at 800 μmol·L$^{-1}$ of Cu treatment but exhibited no significant changes under Zn treatment. Wang et al. (2004) demonstrated that high concentrations of Cu induced several O$_2$ and inhibited CAT activity. Similar to APX, GPX also reduces H$_2$O$_2$ during the oxidation of GSH to GSSG (Stasolla and Yeung 2010). We found that 400 and 800 μmol·L$^{-1}$ of Cu treatment increased the activities of APX and GPX in the leaves, except for the 800 μmol·L$^{-1}$ of Zn treatment which increased the GPX activity, the other Zn concentrations resulted in no significant changes. A study by Yang et al. (2021) reported that excessive Zn inhibits the function of GPX in tobacco leaves.

4. Conclusion

Cu stress significantly reduced the chlorophyll content of the leaves, while Zn stress only reduced the Chl a content. The $F_v/F_m$ decreased significantly under Cu stress but was not affected by Zn treatment. However, the $P_{ABS}$ of the leaves were sensitive to Cu and Zn stress. Both Cu and Zn stress resulted in the weakening of the ability of PQ library to accept electrons, the damage of OEC and the inhibition of the electron transfer from $Q_A$ to $Q_B$. Moreover, Cu stress also dissociated the thylakoids of leaves, but Zn stress did not significantly damage it. In Cu and Zn stressed leaves, the reduction of $RC/CS_m$ significantly increased the $ABS/RC$ and $TR_/RC$ values. Cu stress significantly increased the O$_2$ production rate, H$_2$O$_2$ content, and MDA accumulation in the leaves. However, Zn stress exhibited a minimal effect on the ROS production and oxidative damage in the alfalfa leaves but increased the O$_2$ production rate at the
concentration of 800 μmol·L⁻¹. Cu stress increased the activities of SOD, POD, CAT, APX, and GPX in the leaves; however, leaves adapt to Zn stress by enhancing the activities of SOD and GPX. Thus under Cu stress, the degree of photoinhibition and oxidative damage in alfalfa leaves were significantly higher than under Zn stress.

Disclosure statement
No potential conflict of interest was reported by the author(s).

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References

Ali B, Song WJ, Hu WZ, Luo XN, Gill RA, Wang J, Zhou WJ. 2014. Hydrogen sulfide alleviates lead-induced photosynthetic and ultrastructural changes in oilseed rape. Ecotoxicol Environ Saf. 102:25–33.

Ambrosini VG, Rosa DJ, Melo GWB, Zalamena J, Cella C, Simao DG, Silva LS, Santos HP, Toselli M, Tschirner TL, Brunetto G. 2018. High copper content in vineyard soils promotes modifications in photosynthetic parameters and morphological changes in the root system of ‘Red niagara’ plantlets. Plant Physiol Biochem. 128:89–98.

Ann C, Jiao V, Herman C. 2001. The redox status of plant cells (ASA and GSH) is sensitive to zinc imposed oxidative stress in roots and primary leaves of Phaseolus vulgaris. Plant Physiol Biochem. 39 (7):657–664.

Baker NR. 2008. Chlorophyll fluorescence: a probe of photosynthesis in vivo. Annu Rev Plant Biol. 59(1):89–113.

Baxter A, Mittler R, Suzuki N. 2014. ROS as key players in plant stress signalling. J Exp Bot. 65:1229–1240.

Baycu G, Greve-Kurum N, Moustaka J, Csartoi I, Rognes SE, Moustakas M. 2016. Cadmium-zinc accumulation and photosystem II responses of noccaea caerulescens to Cd and Zn exposure. Environmental Science and Pollution Research. 24(3):2840–2850.

Boojar MMA G. 2007. The copper tolerance strategies and the role of antioxidative enzymes in three plant species grown on copper mine. Chemosphere. 67(11):2138–2147.

Broadley MR, Hammond JP, Zelko I, Lux A, White PJ. 2007. Zinc in plants. New PhytoL. 173(4):677–702.

Bruna A, Urbach W, Dietz KJ. 2010. Compartmentation and transport of zinc in barley primary leaves as basic mechanisms involved in zinc tolerance. Plant Cell Environ. 17(2):153–162.

Bu F. 2019. Physiological and biochemical response of ‘hanfu’ apple seedlings to heavy metal copper stress. Shenyang Agricultural University.

Buapet P, Mohammadi NS, Pernice M, Kumar M, Kuzhiumparambil U, Ralph PJ. 2018. Excess copper promotes photooxidation and modulates the expression of antioxidant-related genes in zostera muelleri. Aquat Toxicol. 207:91–100.

Chaabene Z, Hakim IR, Rotar A, Elleuch A, Mejdoub H, Vandenbulcke F. 2018. Copper toxicity and date palm (Phoenix dactylifera) seedling tolerance: Monitoring of related biomarkers. Environ Toxicol Chem. 37(3):797–806.

Chen JR, Shafi U, Li S, Wang Y, Wu JS, Ye ZQ, Peng DL, Yan WB, Lui D. 2015. Copper induced oxidative stresses, antioxidant responses and phytoremediation potential of moso bamboo (Phyllostachys pubescens). Sci Rep. 5:13554.

Deng F, Yamaji N, Xia J, Ma JF. 2013. A member of the heavy metal P-type ATPase OsHMA5 is involved in xylem loading of copper in rice. Plant Physiol. 163(3):1353–1362.

Elstner FE, Heupel A. 1976. Inhibition of nitrite formation from hydroxylammoniumchloride: a simple assay for superoxide dismutase. Anal Biochem. 70(2):616–620.

Ernst L, Nordenbrand K. 1977. Microsomal lipid peroxidation. Methods in Enzymol. 52(11):302–310.

Essenime J, Govindachary S, Annam S, Bouizid S, Carpentier R. 2012. Enhanced sensitivity of the photosynthetic apparatus to heat stress in digalactosyl-diacylglycerol deficient arabidopsis. Environ Exp Bot. 80:16–26.

Facchinelli A, Sacchi E, Mallen L. 2001. Multivariate statistical and GIS-based approach to identify heavy metal sources in soils. Environ Pollut. 114(3):313–324.

Fan LM, Ma QZ, Liang JQ, Li HF, Wang ET, Wei GH. 2011. Characterization of a copper-resistant symbiotic bacterium isolated from Medicago lupulina growing in mine tailings. Bioresearch Technol. 102(2):703–709.

Foyer CH. 2018. Reactive oxygen species, oxidative signaling and the regulation of photosynthesis. Environ Exp Bot. 154:134–142.

Gong Q, Wang L, Dai TW, Zhou YJ, Kang Q, Chen HB, Li K, Li ZH. 2019. Effects of copper on the growth, antioxidant enzymes and photosynthesis of spinach seedlings. Ecotoxicol Environ Saf. 171:771–780.

Govindjee E. 1995. Sixty-three years since kautsky: chlorophyll a fluorescence: A Signature of Photosynthesis. Haldimann P, Strasser RJ. 1999 Effects of anaerobiosis as probed by the polyphasic chlorophyll a fluorescence rise kinetic in pea (Pisum sativum L.). Photosynth Res. 62(1):67–83.

Halliwell B, Gutteridge JMC. 1985. Free radicals in biology and medicine. Journal of Free Radicals in Biology and Medicine. 1 (4):331–332.

Hammerschmitt RK, Tschirner TL, Facco DB, Silva LOS, Schwalbert R, Drescher GL, Trentin E, Somavilla LM, Kulmann MSS, Silva ICB, et al. 2020. Copper and zinc distribution and toxicity in ‘jade’/‘genovesa’ young peach tree. Sci Hort. 259:1–9.

He GQ, Zhang HB, Liu SQ, Li HQ, Huo YZ, Guo KW, Xu ZS, Zhang HH. 2021. Exogenous γ-glutamyl acid (GABA) induces proline and glutathione synthesis in alleviating Cd-induced photosynthetic inhibition and oxidative damage in tobacco leaves. Journal of Plant Interactions. 16(1):296–306.

Huang Z, Yu L, Zeng C, Yan F, Wu GL. 2018. Soil water storage deficit of alfalfa (Medicago sativa) grasslands along ages in arid area (China). Field Crops Res. 221:1–6.

Kavamura VN, Espósito E. 2010. Biotechnological strategies applied to the decontamination of soils polluted with heavy metals. Biotechnol Adv. 28(1):61–69.

Kupper H, Kupper FC, Spiller M. 1998. In situ detection of heavy metal substituted chlorophylls in water plants. Photosynthesis Res. 58(2):123–133.

Lee HJ, Lee JH, Wi S, Jang Y, An S, Choi CK, Jang S. 2021. Exogenously applied glutamic acid confers improved yield through increased photosynthesis efficiency and antioxidant defense system under chilling stress condition in Solanum lycopersicum L. cv. dataeorg Dia. Sci Hortic. 277(3):109617.

Lee I, Sales J, Martins LL, Mourato MP. 2021. Response to stress induced by different potentially toxic elements (As, Cd, Cu and Na) in rapsweed leaves. Plant Physiology Reports. 26(3):478–490.

Li L. 2019. Physio-biochemical and molecular mechanism of exogenous brassinosteroids in regulating growth of Brassica napus under copper and chromium stress. Zhejiang University.

Li PM, Gao HY, Strasser RJ. 2005. Application of the fast chlorophyll fluorescence induction dynamics analysis in photosynthesis study. Acta Photophysiolegica Sinica. 31(6):359.
