EXOGENOUS GLUTATHIONE REDUCES THE DAMAGE INDUCED BY CADMIUM IN CUCUMBER SEEDLINGS

GLUTATIONA EXÓGENA REDUZ O DANO INDUZIDO POR CADMIO EM PLANTULAS DE PEPINO

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ABSTRACT: Cadmium (Cd) toxicity is a worldwide problem for crop production. The present work used hydroponic experiments to investigate the ameliorating effects and physiological mechanisms of glutathione (GSH) mitigation of Cd toxicity in cucumber seedlings. The results revealed that Cd was mainly accumulated in roots of cucumber, 100 µM GSH pretreatment in 50 µM Cd solution significantly recovered Cd-induced growth inhibition, improved photosynthetic and chlorophyll fluorescence performance. Moreover, external GSH obviously depressed hydroxyl free radical (·OH) and malondialdehyde accumulation, increased the total antioxidant capacity in cucumber exposed to Cd. Results indicated that pre-treatment of GSH can alleviate Cd toxicity by reducing Cd uptake and ROS accumulation, reduce the negative consequences of oxidative stress caused by Cd toxicity, moreover protect photosynthetic machinery from damaging, balance nutrients and antioxidants in cucumber.


INTRODUCTION

Heavy metal contamination has become one of the most important environmental problems worldwide. Cd represents a major environmental pollutant and has been classified as seventh out of 275 compounds in the priority list of hazardous materials by the Comprehensive Environmental Response, Compensation and Liability Act (ATSDR, 2008). In soil, Cd even in low concentration could enter crop plants and preferentially concentrate in their edible parts; hence, Cd is also a toxic trace pollutant for humans, animals and plants via food chain (BUR et al., 2010). Large number of animals exposed to high concentration of Cd have been found suffering from mutagenic, carcinogenic and teratogenic (DEGRAEVE, 1981). Once entering in plants, Cd can cause photosynthesis reduction, nutrient uptake decrease, and visible injury symptoms, such as chlorosis, growth inhibition, root tips browning, and finally death (KAHLE, 1993; WANG et al., 2011; LIU et al., 2015). Correspondingly, it is urgently necessary to develop approaches to prevent Cd accumulation in plant edible parts so as to alleviate health risks associated with exposure to highly Cd-containing food.

The presence of Cd can result in excessive generation of reactive oxygen species (ROS) which could cause cell death due to oxidative stress such as membrane lipid peroxidation, enzyme inhibition, protein oxidation and damage to nucleic acids (GILL; TUTEJA, 2010; HOSSAIN et al., 2010; GILL et al., 2011a). To repair the Cd-induced inhibitory effects of ROS, plants employ a ROS-detoxifying antioxidant defense machinery which includes non-enzymatic (reduced glutathione (GSH), AsA, a -tocopherol and carotenoids) and enzymatic (SOD, CAT, APX, GR, MDHAR, DHAR, GPX and GST) antioxidants (GILL; TUTEJA, 2010; HOSSAIN et al., 2010; GILL et al., 2011b; LIN et al., 2012; SUN et al., 2014; LIU et al., 2015). Moreover, GSH and oxidized glutathione (GSSG) are particularly important in scavenging ROS based on the GSH–GSSG reaction and the ascorbate–glutathione cycle (NOCTOR et al., 2012). Exposure to Cd provoke prominent responses of antioxidative systems, but the direction of the response dependends on the plant species, plant organ, and the intensity of the Cd stress (SCHÜTZENDÜBEL; POLLE, 2002 ).

GSH, a naturally occurring tripeptide, is a constituent of the cellular defense mechanism of the body against various exogenous as well as endogenously produced xenobiotics, oxyradicals, salinity, acidity, and metal cations (FERGUSON; BOOTH, 1998; WU et al., 2003). Chen et al. (2010) and Wang et al. (2011) found that exogenous GSH was one of the favorable antioxidants in the defense system against Cd stress in barley. More...
Exogenous glutathione reduces

interestingly, Chao et al. (2009) reported that rice seedlings pre-treated with 1 mM exogenous GSH showed higher GSH level in leaves and subsequently enhanced Cd tolerance. In our previous study on maize seedlings, pre-treated with 100 µM GSH for 24 hours significantly alleviated 50 µM Cd-induced growth inhibition and dramatically diminished leaf H₂O₂ and root malondialdehyde (MDA) accumulation, significantly decreased Cd concentration, counteracted Cd-induced alterations of certain microelements and antioxidant enzymes (SUN et al., 2013). Besides its role in the ascorbate - glutathione cycle, glutathione can act as a first defense line against metal toxicity through complexing metals before phytochelatins (PCs) reaches to an effective level (SINGHAL et al., 1987). In addition, GSH is also an immediate substrate for synthesis of PCs (ZENK, 1996). Therefore, the question arises whether and/or how external GSH could act as a regulator in preventing Cd stress in cucumber seedlings.

Hence, the current work was conducted using a hydroponic system to investigate whether exogenous GSH is able to alleviate Cd toxicity in cucumber seedlings, aimed to find an valid way to reduce Cd toxicity risks for sustainable safe production and to explore the underlying mechanism in alleviating cucumber Cd toxicity by external GSH application.

MATERIAL AND METHODS

Plant growth and treatments

Healthy seeds of cucumber variety Jinyan 4 were germinated in sterilized moist sand and grown for 10 days in an incubator with a 16 h photoperiod (180 µmol m⁻² s⁻¹) at 22°C to 25°C in Taiyuan university of science and technology, Taiyuan, China, and the relative humidity was 70%. After 10 days, the uniform healthy seedlings were selected and transplanted to 5 L containers with a volume of 4.5-L basal nutrient solution (JANICKA-RUSSAK et al., 2012), the solution pH was checked for 5.6±0.1, the solution was continuously aerated with pumps. And treatments were conducted on the 6th day after transplanting: (1) control, basal nutrient solution; (2) GSH, 100 µM GSH was added on the 5th day after transplanting and on the next day (6th day, i.e. after 24 hours pre-treated with GSH) replaced with basal nutrient solution; (3) Cd, as 50 µM CdCl₂; and (4) Cd+GSH, 24 h pre-treated with 100 µM GSH+50 µM Cd, GSH was added on the 5th day after transplanting and on the second day replaced with Cd. And seven plants per treatment in 3 replicates were made. All plants were sampled and determined after 5 days Cd treatment.

Chlorophyll contents, photosynthesis and chlorophyll fluorescence parameters analysis

The 2nd fully expanded leaves (The average area was 6.2 cm²) of plants were used for measurement of the chlorophyll content (measured as SPAD (Soil Plant Analysis Development) values), chlorophyll fluorescence and photosynthetic parameters. SPAD value was determined using a chlorophyll meter (SPAD-502, Konica Minolta Sensing, Japan). The net photosynthetic rate (Pn), stomatal conductance (Gs), transpiration rate (Tr) and intercellular CO₂ concentration (Ci) were measured using a Li-6400 portable photosynthesis system (LI-COR, Lincoln, NE, USA). The chlorophyll fluorescence parameters were measured with an IMAGINGPAM (chlorophyll fluorometer) system according to Zhang et al. (2015).

Plant growth and biomass, and element determination

The height and root length of each plant were measured after 5 days Cd treatment, and then all cucumber seedlings were uprooted and separated into leaves, stems and roots. For the determination of microelement (Cd, Zn, Cu) concentration, all the separated samples were dried and digested according to our previous method (SUN et al., 2014), and the detailed procedure has been described elsewhere (LIU et al., 2015).

OH, lipid peroxidation, and antioxidant capacity determination

The fresh samples were directly used for the hydroxyl free radical (·OH) contents using a ·OH detection kit (Jiancheng Bioengineering Institutes, China) in accordance with the manufacturer’s instructions. The level of lipid peroxidation was quantitated by the amount of malondialdehyde (MDA) according to Wu et al. (2003). The antioxidant capacity was determined using cupric reducing antioxidant capacity (CUPRAC) assay according to our previous study (LI et al., 2015), and the final results were given as mg gallic acid equivalents (GAE).

Statistical analysis

All data were means of three replicates and analysed with Data Processing System statistical software package using ANOVA test (TANG Q; FENG, 1997), differences among treatments were
evaluated by the Duncan’s Multiple Range Test (SSR) at significance level of \( P \leq 0.05 \).

**RESULTS AND DISCUSSION**

**Effect of exogenous GSH pretreatment on Cd toxicity and Cd concentration in cucumber seedlings**

As compared with control, cucumber plants exposure to 50 \( \mu \text{M} \) Cd after 5 days induced significant decrease SPAD value, plant height, root length and biomass (Table 1), and the general symptoms of Cd exposure to plants was leaf chlorosis (SUN et al., 2014), which was consistent with the present results. Pretreatment with 100 \( \mu \text{M} \) GSH for 24 h before 50 \( \mu \text{M} \) Cd stress (Cd+GSH) significantly alleviated Cd-induced growth inhibition, i.e. after 5 days Cd+GSH treatment, SPAD value, plant height and root length increased significantly by 12.8%, 11.5% and 21.7% compared with Cd alone treatment.

**Table 1.** Effect of Cd and GSH in nutrient media on SPAD value, plant height, root length and biomass of cucumber seedlings exposed to Cd for 5 days.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>SPAD value</th>
<th>Plant height (cm)</th>
<th>Root length (cm)</th>
<th>Leaf DW (mg plant(^{-1}))</th>
<th>Stem DW (mg plant(^{-1}))</th>
<th>Root DW (mg plant(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>28.0(^a)</td>
<td>22.5(^a)</td>
<td>18.7(^a)</td>
<td>75.1(^a)</td>
<td>45.4(^a)</td>
<td>11.5(^a)</td>
</tr>
<tr>
<td>GSH</td>
<td>27.4(^a)</td>
<td>21.3(^a)</td>
<td>17.2(^a)</td>
<td>72.0(^a)</td>
<td>43.7(^a)</td>
<td>10.4(^a)</td>
</tr>
<tr>
<td>Cd</td>
<td>23.4(^b)</td>
<td>19.2(^b)</td>
<td>12.0(^b)</td>
<td>56.3(^b)</td>
<td>32.2(^b)</td>
<td>7.8(^b)</td>
</tr>
<tr>
<td>Cd+GSH</td>
<td>26.4(^a)</td>
<td>21.4(^a)</td>
<td>14.6(^b)</td>
<td>65.1(^b)</td>
<td>38.2(^b)</td>
<td>9.5(^a)</td>
</tr>
</tbody>
</table>

*Means followed by same letter in columns do not differ statistically among themselves by Duncan test (p > 0.05). DW, dry weight. Control, GSH, Cd and Cd+GSH correspond to basic nutrition solution (BNS), BNS+100 \( \mu \text{M} \) GSH, BNS+50 \( \mu \text{M} \) Cd, and BNS+50 \( \mu \text{M} \) Cd +100 \( \mu \text{M} \) GSH, respectively.*

It has been shown that Cd was more accumulated in roots than in the aerial parts (leaves and stems) after Cd treatment. Furthermore, GSH pretreatment (Cd+GSH) suppressed Cd uptake, and leaf, stem and root Cd concentrations reduced by 27.5%, 29.8% and 33.1%, respectively, when compared with those in Cd alone treatment (Table 2). No significant differences were observed between GSH pretreatment and control which were below the detection limit. The results suggested a practical potential for exogenous GSH pretreatment as an intervention strategy in mitigating Cd stress and reducing Cd uptake and translocation in cucumber plants.

**Table 2.** Effect of Cd and GSH pretreatment on element concentrations in leaves, stems and roots of cucumber seedlings.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Leaf Cd</th>
<th>Zn</th>
<th>Cu</th>
<th>Stem Cd</th>
<th>Zn</th>
<th>Cu</th>
<th>Root Cd</th>
<th>Zn</th>
<th>Cu</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>-</td>
<td>134.8(^a)</td>
<td>30.9(^b)</td>
<td>-</td>
<td>248.1(^a)</td>
<td>59.4(^a)</td>
<td>-</td>
<td>550.4(^a)</td>
<td>63.5(^a)</td>
</tr>
<tr>
<td>GSH</td>
<td>-</td>
<td>130.2(^a)</td>
<td>29.2(^b)</td>
<td>-</td>
<td>242.2(^a)</td>
<td>55.4(^a)</td>
<td>-</td>
<td>542.4(^a)</td>
<td>68.9(^a)</td>
</tr>
<tr>
<td>Cd</td>
<td>43.6(^b)</td>
<td>87.5(^b)</td>
<td>20.2(^c)</td>
<td>183.3(^b)</td>
<td>159.7(^b)</td>
<td>33.7(^b)</td>
<td>904.8(^a)</td>
<td>470.9(^b)</td>
<td>49.0(^b)</td>
</tr>
<tr>
<td>Cd+GSH</td>
<td>31.6(^b)</td>
<td>92.1(^b)</td>
<td>44.3(^a)</td>
<td>128.7(^b)</td>
<td>163.5(^b)</td>
<td>65.8(^a)</td>
<td>604.9(^b)</td>
<td>502.8(^ab)</td>
<td>66.0(^a)</td>
</tr>
</tbody>
</table>

Values are in mg kg\(^{-1}\) DW. –, not detected.

In plants, Cd stress can disturb the metabolism of some micronutrients. The interactions of Cd and metal nutrients have been reported in some upland crops, such as wheat (ZHANG et al., 2002), barley (WANG et al., 2011; MUHAMMAD et al., 2012; SUN et al., 2014), and tomato (SMITH; BRENNAN, 1983). However, the interactions between Cd and other nutrients were complicated and quite different with species. Our previous study found that there was a significantly negative correlation between Zn, Cu, or Mn concentration and Cd concentration in barley plants (SUN et al., 2014). The same effects were observed in the present experiment. Cucumber plants exposed to 50 \( \mu \text{M} \) Cd significantly reduced Zn and Cu concentrations in all tissues, external GSH noticeably recovered leaf, stem and root Cu, and leaf Cu concentration was higher than control level, e.g. leaf Cu concentration in Cd+GSH treatment being 2.19 fold higher than Cd alone treatment (Table 2), indicating that GSH can mitigate Cd toxicity in cucumber through balancing the elements metabolism.
Photosynthetic and chlorophyll fluorescence parameters in leaves of cucumber seedlings

Earlier investigations have demonstrated that there was a notable reduction in $Pn$ by Cd treatment in many plant species (CAI et al., 2011; BASZYNSKI et al., 1980). In the present research, the results were also consistent with these observations, cucumber plants exposed to 50 µM Cd showed a sharp decrease in $Pn$, being 28.5% lower compared with the control condition (Table 3), which accompanied with marked reduction in $Tr$ and $Gs$. Furthermore, the $Ci$ increased significantly after Cd treatment, while Cd-GSH suppressed Cd-induced increase, therefore the inhibition of photosynthetic processes by Cd in cucumber was due to nonstomatal restriction. In addition, GSH pretreatment (Cd+GSH) improved cucumber photosynthetic ability inhibited by Cd stress, with the $Pn$ increased significantly by 21.0%, which might likely link to protective mechanisms that maintain the integrity of the photosynthetic machinery.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>$Pn$ (µmCO$_2$ m$^{-2}$ s$^{-1}$)</th>
<th>$Tr$ (mM H$_2$O m$^{-2}$ s$^{-1}$)</th>
<th>$Gs$ (mM H$_2$O m$^{-2}$ s$^{-1}$)</th>
<th>$Ci$ (µM M$^{-1}$)</th>
<th>CO$_2$</th>
<th>$F_0$</th>
<th>$F_{v}/F_{m}$</th>
<th>$Y$(NO)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>16.2$^{a}$</td>
<td>2.4$^{a}$</td>
<td>110.3$^{a}$</td>
<td>329$^{a}$</td>
<td>0.055$^{b}$</td>
<td>0.849$^{b}$</td>
<td>0.155$^{b}$</td>
<td></td>
</tr>
<tr>
<td>GSH</td>
<td>16.1$^{a}$</td>
<td>2.6$^{a}$</td>
<td>109.4$^{a}$</td>
<td>326$^{a}$</td>
<td>0.050$^{a}$</td>
<td>0.860$^{a}$</td>
<td>0.140$^{a}$</td>
<td></td>
</tr>
<tr>
<td>Cd</td>
<td>11.6$^{b}$</td>
<td>1.9$^{b}$</td>
<td>75.4$^{b}$</td>
<td>415$^{a}$</td>
<td>0.071$^{a}$</td>
<td>0.820$^{b}$</td>
<td>0.173$^{a}$</td>
<td></td>
</tr>
<tr>
<td>Cd+GSH</td>
<td>14.0$^{b}$</td>
<td>2.1$^{ab}$</td>
<td>85.4$^{b}$</td>
<td>377$^{b}$</td>
<td>0.053$^{b}$</td>
<td>0.858$^{b}$</td>
<td>0.142$^{b}$</td>
<td></td>
</tr>
</tbody>
</table>

$Pn$ net photosynthetic rate, $Tr$ transpiration rate, $Gs$ stomatal conductance, $Ci$ intercellular CO$_2$ concentration.

The initial fluorescence ($F_0$) and the maximum efficiency of photosystem II photochemistry ($F_{v}/F_{m}$) were widely used as reliable diagnostic indicators of photoinhibition (Krause, 1988). $F_{v}/F_{m}$ was decreased exposing to 50 µM Cd treatment, however, $F_0$ and quantum yield of nonregulated energy dissipation $Y$(NO) increased significantly (Table 3), similar results were observed in barley, rice and tobacco of $F_{v}/F_{m}$ value being decreased significantly under Cd stress (WANG et al., 2011; CAI et al., 2011; LIU et al., 2015). The $F_0$ was also the fluorescent when the reaction center of photosystem II (PSII) was all open, and the increase in $F_0$ under Cd stress indicated the injury of PSII. Considerable change of $Y$(NO) under Cd stress indicated there was severe irreversible photodamage to PSII (KRAMER et al., 2004). On the other hand, the decrease of $F_{v}/F_{m}$ under Cd stress also indicated that the photochemistry of PSII and its ability to reduce the primary acceptor Q$_A$ were also affected by Cd, and they were sensitive to environmental changes, which affected efficiency to capture excitation energy by open PSII reaction center (BABANI; LICHTENTHALER, 1996). On the contrary, GSH pretreatment (Cd+GSH) improved the $F_{v}/F_{m}$ ratio back to control level, and obviously suppressed Cd-induced increase in the $F_0$ and $Y$(NO) by 25.4% and 17.9%, respectively, indicating GSH regulated photosynthesis improvement partly owing to the chlorophyll synthesis increase and the protection of photosystem II reaction center, which were significant for improving Cd tolerance in cucumber.

GSH eliminated Cd-induced over-accumulation of MDA, ·OH, and elevated Cd-depressed antioxidant capacity in cucumber seedlings

Oxidative stress was a central part of abiotic and biotic stresses, ROS such as O$_2$•-, H$_2$O$_2$ and ·OH were often produced in large quantities by plants during various stress responses (CHEN et al., 2010; VIEHWEGER, 2014), in order to protect against oxidative stress, plants have evolved enzymatic and non-enzymatic ROS scavenging systems.

Our results showed that Cd-induced accumulation of ·OH and MDA in leaves, stems and roots of cucumber seedlings, which were markedly reduced by GSH pretreatment (Fig. 1). GSH was one of the non-enzymatic components acting as an antioxidant, and it was involved directly in the reduction of most ROS (NOCTOR and FOYER, 1998); additionally, GSH played a key role in the antioxidative defense system by regenerating other potential water-soluble antioxidants like AsA via the AsA-GSH cycle (FOYER and HALLIWELL, 1976). In present study, GSH pretreatment significantly reduced MDA content although its value still lower than that in control, for example, in leaves, stems and roots, it decreased by 31.8%, 28.2% and 13.6% under Cd+GSH, respectively, compared with the Cd-alone treatment (Figure 1A, B and C). The variation of ·OH content showed a similar trend to that of MDA (Figure 1D, E and F). Antioxidant capacity expressed as CUPRAC value was significantly reduced in all tissues under Cd treatment compared with control, and especially in

roots, reduced by 72.2%. Importantly, CUPRAC value increased under Cd +GSH condition although the value was lower than that observed in the control (Figure 1G, H and I).

Figure 1. Effect of external GSH pretreatment on MDA accumulation, ·OH content and cupric reducing antioxidant capacity (CUPRAC) in leaves, stems and roots of cucumber seedlings exposed to Cd for 5 days. FW and GAE represent fresh weight and gallic acid equivalent, respectively.

CONCLUSIONS

The protective role of GSH in alleviation of Cd-induced growth inhibition in cucumber was complicated.

The mechanism involved in the prevention of Cd stress is mainly linked to the decreased Cd in leaves, stems and roots and dramatically depressed ·OH and MDA accumulation, and elevated Cd-depressed antioxidant capacity in cucumber seedlings compared with Cd treatment. This result also suggests that GSH has antioxidant properties or that GSH activates protective mechanisms that can alleviate oxidative stress in non-enzymatic ways. On the other hind, GSH ameliorated Cd-induced reduction in chlorophyll content, improved photosynthetic and chlorophyll fluorescence performance. Furthermore, the alleviating effect of GSH was associated with balanced nutrient elements in cucumber plants. These results may facilitate a better understanding of the mechanisms involved in GSH-mediated tolerance to Cd stress in cucumber.

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**RESUMO:** A toxicidade do Cádmio (Cd) é um problema mundial para a produção de plantas olerícolas. O presente trabalho com o uso experimentos hidropônicos objetivou investigar a atenuação dos efeitos do cádmio por meio de mecanismos envolvendo a glutationa (GSH) e redução da toxicidade em plântulas de pepino submetidas ao Cd. Os resultados revelaram que o Cd acumulou principalmente nas raízes do pepino. Na dose de 100 µM GSH-50 esta reduziu a concentração em µM Cd na solução e a mesma melhora a ou reduz a inibição do crescimento bem como melhora o desempenho e a fluorescência clorofílica. Além disso, obviamente, radical Livre hidroxila GSH externa (·OH) aumenta a acumulação do malondialdeído e aumento da capacidade antioxidante total no pepino expostos ao Cd. Os resultados indicaram que o pré-tratamento de GSH pode reduzir a toxicidade, reduzindo a captação Cd e acumulação ROS, reduzir os efeitos negativos do stress oxidativo reduzindo a sua toxicidade, além de proteger o equilíbrio da disponibilidade de nutrientes e antioxidantes.

**PALAVRAS-CHAVE:** Cádmio. Glutationa. Espécies reativas de oxigênio. Fotossíntese. Pepino (*Cucumis sativus* L)

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