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Effect of cadmium on photosynthetic pigments, lipid peroxidation, antioxidants, and artemisinin in hydroponically grown *Artemisia annua*

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Abstract

The effects of different cadmium (Cd) concentrations (0, 20, 60, and 100 μmol/L) on hydroponically grown *Artemisia annua* L. were investigated. Cd treatments applied for 0, 4, 12, 24, 72, 144, 216, and 336 hr were assessed by measuring the changes in photosynthetic pigments, electrolyte leakage, malondialdehyde (MDA) and antioxidants (ascorbic acid and glutathione), while the artemisinin content was tested after 0, 12, 144, 216, and 336 hr. A significant decrease was observed in photosynthetic pigment levels over time with increasing Cd concentration. Chlorophyll *b* levels were more affected by Cd than were chlorophyll *a* or carotenoid levels. The cell membrane was sensitive to Cd stress, as MDA content in all treatment groups showed insignificant differences from the control group, except at 12 hr treatment time. Ascorbic acid (AsA) content changed slightly over time, while glutathione (GSH) content took less time to reach a maximum as Cd concentration increased. Cd was found to promote synthesis and accumulation of artemisinin, especially at concentrations of 20 and 100 μmol/L. In conclusion, Cd stress can damage to photosynthetic pigments, and vigorously growing *A. annua* showed a strong tolerance for Cd stress. Appropriate amounts of added Cd aided synthesis and accumulation of artemisinin.

Key words: cadmium; photosynthetic pigments; oxidative stress; artemisinin; *Artemisia annua*
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Introduction

Heavy metal contamination is a serious global environmental problem. With the wide use of cadmium (Cd) in modern industrial technology, especially smelting, electroplating, batteries, pigments, and industrial wastewater, the contribution of human activities to cadmium in the soil greatly exceeds natural release (Nriagu and Pacyna, 1988). It is generally thought that Cd is a toxic metal with no biological function, except in some marine diatoms (Lane and Morel, 2000; Lane et al., 2005). Cd in the soil can reach the human body through the food chain and can bioaccumulate (Florijn and Beusichem, 1993). Cd in the food chain can increase the incidence of human diseases, and it has a toxic effect on plants, animals, and microorganisms (Nogawa, 1987).

Cd stress can induce physiological changes including inhibiting seed germination, reducing plant growth (Huang et al., 1974; Larbi et al., 2002), decreasing photosynthetic efficiency (Ciscato et al., 1999; Ekmekci et al., 2008; Uraguchi et al., 2006), and changing the uptake rates for some ions (Shi et al., 2003). Cd is not an essential nutrient for plants, but it is readily taken up and both directly and indirectly affects several metabolic activities in different cell compartments, especially chloroplasts (Ekmekci et al., 2008).

Cd stress is one environmental stress that triggers reactive oxygen species (ROS) levels to increase dramatically (Lefèvre et al., 2010). One of the most damaging effects is lipid membrane peroxidation and resultant ion leakage (Gupta et al., 2009). To control ROS levels in plants, an antioxidant system is needed to protect the cell (Dinakar et al., 2008). The antioxidant system consists of the non-enzymatic system (ascorbic acid AsA, glutathione GSH) and antioxidant enzymes, such as superoxide dismutase (SOD), catalase (CAT) and peroxidase (POD) (Foyer et al., 1994; Noctor and Foyer, 1998). In addition, ROS can act as signals to activate plant defense systems (Mittler, 2002). They can rapidly activate protective mechanisms that play an important role in alleviating the hazardous effects of stress (Liu et al., 2007).

AsA and GSH are important antioxidants, and are major components of the non-enzymatic system for free radical scavenging. They can control H_2O_2 levels in cells through the AsA-GSH cycle with ascorbate peroxidase (APX) and glutathione reductase (GR) (Liu et al., 2007). Moreover, GSH is very important for plant resistance to environmental stress, especially Cd stress (Ortega-Villasante et al., 2005). It is well known that the synthesis and recovery capability of GSH confers stress resistance to plants (Horemans et al., 2007; Metwally et al., 2005).

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In most developed countries, Asian herbal medicines are becoming more and more popular. For Chinese remedies, traditional Chinese medicines (TCMs) have been reported to be contaminated by heavy metals. Cadmium is often implicated as one of the contaminants. However, under the condition of keeping the plant alive, the appropriate amount of heavy metal can promote the synthesis and accumulation of secondary metabolites which are considered to be the main active components in TCMs. It is significant to investigate the changes of the physiology and the secondary metabolites in medical plants under various Cd treatments.

Artemisia annua is an effective antimalarial herb, used against malaria and heat stroke in traditional Chinese medicine (Liu et al., 2002). Artemisinin, the main active component, is a secondary metabolite in *A. annua* (Liu et al., 1998). Secondary metabolites play major roles in adaptation to continuously changing environments and overcoming environmental stresses.

In order to get more information on the *A. annua* responses to Cd under hydroponic conditions, in this research, the effects of Cd on photosynthetic pigments, relative conductivity, MDA (lipid peroxidation), content of AsA and GSH, and artemisinin content were analyzed under various Cd treatments.

1 Materials and methods

1.1 Materials preparation and collection

A. annua seeds were obtained from Guangxi Medicinal Botanical Garden, Guangxi Province, China. Seeds were surface sterilized with 10% hydrogen peroxide and rinsed with double-distilled water. Seeds were germinated and grown in sterilized sand. Seedlings were raised in half-strength Hoagland nutrient solution. When the seedlings were 4–5 cm high (at about forty five days old), well-grown seedlings of a similar size were transplanted into hydroponic pots (five seedlings per pot), containing 500 mL Hoagland nutrient solution. Seedlings were fixed onto the pots by wrapping the stems with clean sterilized sponges. The solution was changed weekly. Hydroponic units were aerated through a system of flexible plastic tubes immersed in the solution, and connected to air pumps. After one month of growth, the solution was given various concentrations of Cd, at pH 5.8. Solutions were changed once every 3 days. Treatments used were 0, 20, 60, and 100 μmol/L of $Cd(NO_3)_2{\cdot}4H_2O$. Each treatment was conducted nine times (each pot was considered a repeat). The experiment was conducted in a greenhouse with a photosynthetic photon flux density (PPFD) of 260–350 lx measured at leaf height, 40%–45% relative humidity, and a 10 hr 25°C/14 hr 18°C, day/night regime.

Upper mature leaves of similar size were used for determination of photosynthetic pigments, electrolyte leakage, MDA, and antioxidants (AsA and GSH), after 0, 4, 12, 24, 72, 144, 216, and 336 hr. Artemisinin levels were measured at 0, 12, 144, 216, and 336 hr, in *A. annua* cleaned with distilled water. *A. annua* was separated into above- and below-ground parts. The above-ground parts were crushed through a 180 micron mesh sieve after drying at low temperature.

1.2 Photosynthetic pigment assays

The leaves (1 g fresh weight (fw)) were homogenized in 35 mL of 80% acetone. After centrifugation for 10 min at 3500 ×*g*, the absorbences of the supernatant were measured at 663, 646, and 470 nm with a UV-Vis spectrophotometer (T6, Persee, China). Levels of chlorophyll *a* (Chl-*a*), chlorophyll *b* (Chl-*b*), total chlorophyll and carotenoids were measured using the method described by Wellburn (2004).

1.3 Electrolyte leakage experiments

Electrolyte leakage was measured as described by Liu et al. (2008). Leaf discs 1 cm in diameter (10 discs from at least three plants) were immersed in 2 mL deionized water at room temperature for at least 12 hr. Conductivity was then measured. Tubes containing leaf discs were boiled for 30 min, cooled, and conductivity was remeasured. Electrolyte leakage was calculated as the percentage of the initial value over the final value.

1.4 Malondialdehyde levels

MDA content was measured as described by Heath and Packer (1968), with some modifications. Leaves (0.5 g fw) were homogenized in 5 mL of 0.1% trichloroacetic acid. The homogenate was centrifuged at 10,000 ×*g* for 5 min. The reaction mixture was 1 mL of the supernatant and 4 mL of 0.6% thiobarbituric acid in 20% trichloroacetic acid. The mixture was incubated in boiling water for 30 min, and then the reaction was terminated by transference to an ice bath. The absorbences of the mixture were measured at 532 nm and adjusted for nonspecific absorption at 450 and 600 nm (Tang et al., 2010).

1.5 Ascorbic acid and glutathione levels

AsA content was determined as described by Tanaka et al. (1985) and GSH content was determined as described by Ellman (1959).

1.6 Artemisinin analysis by high performance liquid chromatography

Leaf powder from *A. annua* (0.5 g) was weighed in a conical flask with a stopper. After adding 25 mL of petroleum ether (60–90°C), the powder was exposed to ultrasonic extraction (100 W, 40 kHz) for 50 min. The extraction solution was filtered after it was cooled to room temperature. The residue obtained was dried by distillation in the rotary evaporator (R-200, BÜCHI Rotavapor, Switzerland), and then dissolved in methanol to a fixed volume (5 mL). A sample was taken for HPLC analysis (Zhang et al., 2007).

Methods for analyzing artemisinin have been described by Özgüven et al. (2008); we used these with some modifications. The analysis was performed on a Waters 2965 Separations Module (Alliance HPLC, Waters, USA) equipped with a Waters 2996 Photodiode Array Detector.

Chromatographic data were acquired and processed with Waters Millennium 32 Software. The column was an Agilent TC-C18 (250 mm × 4.6 mm, 5 μm); the column temperature was 25°C. The mobile phase was formic acid (0.1%, *V*/*V*) and acetonitrile (50:50, *V*/*V*) by isocratic elution. Detection of UV absorbance was at 209 nm, the flow rate was 1.0 mL/min, and the injection volume was 10 μL.

1.7 Statistical analysis

Each experiment was conducted at least three times, independently. We used Duncan's multiple range test (DMRT) to determine significant differences between control and treatment groups, and examine levels of significance. The interaction of Cd concentration and time was analyzed by using the two-way ANOVA procedure, with multi-comparisons by Tukey's test. All results were expressed as means. Statistical analyses were performed using SPSS software.

2 Results and discussion

The pigment content was significantly affected by Cd (Table 1). The Chl-*a*, Chl-*b*, total chlorophyll, and carotenoid levels decreased with increasing Cd concentration, to 336 hr.

Chl-*a* content in the control group and at 20 μmol/L Cd concentration decreased by 31.34% and 48.94% in 336 hr. At 60 μmol/L Cd concentration, Chl-*a* content showed only slight changes between 4 and 72 hr, then began to fall obviously at 144 hr, with a decrease of 76.76% at 336 hr. Also, for the 100 μmol/L Cd concentration, levels showed a continuous drop starting from 12 hr, and falling by 72.89% in 336 hr.

In the control group and at 20 μmol/L Cd concentration, Chl-*b* content was decreased by 51.69% and 61.86% at 336 hr. Chl-*b* content at 60 μmol/L Cd concentration decreased slightly between 4 and 72 hr but fell significantly starting at 144 hr, decreasing by 86.44% in 336 hr. At 100 μmol/L Cd concentration, Chl-*b* content fell by 83.90% in 336 hr.

Total chlorophyll content in the control group decreased by 37.16% and at 20 μmol/L Cd concentration decreased by 52.87% in 336 hr. At 60 μmol/L Cd concentration, total chlorophyll decreased slightly between 4 and 72 hr, and fell by 76.76% in 336 hr. At 100 μmol/L Cd concentration, total chlorophyll content dropped sharply starting from 144 hr and fell by 76.31% in 336 hr.

Carotenoid content in the control group and 20 μmol/L Cd treatment decreased by 38.46% and 55.77% in 336 hr. For the 60 μmol/L Cd concentration, carotenoid content showed a slight drop between 4 and 72 hr but fell significantly from 144 hr, decreasing by 79.30% in 336 hr. For the 100 μmol/L Cd concentration, carotenoid content fell by 73.08% in 336 hr (Table 1). Chl-*a*/Chl-*b* ratios in the control and treatment groups (20, 60, and 100 μmol/L) were raised by 42.15%, 62.81%, 71.49%, and 75.62% respectively.

The Chl-*a*, Chl-*b*, total chlorophyll, and carotenoid content in the treatment groups all exhibited a decrease greater than 30%; this was not the case in the control group. The decrease was greater than 70% for the 60 and

Table 1 Effects of Cd concentrations and treatment time on Chl-*a*, Chl-*b*, total chlorophyll, carotenoids, and Chl-*a*/Chl-*b*

Cd conc. (μmol/L)	Treatment time (hr)							
	0	4	12	24	72	144	216	336
Chl-*a* (mg/g fw)								
Control	2.837 ± 0.139 Aa	2.640 ± 0.144 ABa	2.535 ± 0.300 Aab	1.760 ± 0.156 Bd	1.832 ± 0.071 Bcd	2.178 ± 0.119 Abc	2.122 ± 0.075 Acd	1.951 ± 0.081 Acd
20	2.837 ± 0.139 Aa	2.625 ± 0.178 ABa	2.366 ± 0.038 Ab	1.856 ± 0.095 Bc	1.854 ± 0.052 Bc	1.497 ± 0.126 Bd	1.264 ± 0.081 BCd	1.445 ± 0.078 Bd
60	2.837 ± 0.139 Aa	2.331 ± 0.054 Bb	2.455 ± 0.026 Ab	2.376 ± 0.086 Ab	2.375 ± 0.186 Ab	1.197 ± 0.136 Cc	1.438 ± 0.141 Bc	0.665 ± 0.087 Cd
100	2.837 ± 0.139 Aa	2.799 ± 0.124 Aa	1.816 ± 0.181 Bb	1.570 ± 0.160 Bbc	1.479 ± 0.184 Bbcd	1.254 ± 0.062 Ccd	1.139 ± 0.082 Cd	0.767 ± 0.119 Cf
Chl-*b* (mg/g fw)								
Control	1.177 ± 0.110 Aa	0.999 ± 0.059 ABab	0.966 ± 0.153 Ab	0.652 ± 0.072 Bc	0.701 ± 0.039 ABc	0.747 ± 0.080 Ac	0.662 ± 0.049 Ac	0.566 ± 0.018 Ac
20	1.177 ± 0.110 Aa	0.980 ± 0.078 ABab	0.836 ± 0.016 ABbc	0.645 ± 0.036 Bcde	0.703 ± 0.025 ABcd	0.564 ± 0.204 Ade	0.322 ± 0.032 Cf	0.447 ± 0.037 Bef
60	1.177 ± 0.110 Aa	0.846 ± 0.034 Bb	0.892 ± 0.004 Ab	0.946 ± 0.038 Aab	0.841 ± 0.092 Ab	0.488 ± 0.230 Ac	0.437 ± 0.060 Bc	0.163 ± 0.039 Cd
100	1.177 ± 0.110 Aa	1.039 ± 0.080 Aa	0.658 ± 0.073 Bb	0.585 ± 0.055 Bb	0.567 ± 0.069 Bb	0.343 ± 0.028 Ac	0.328 ± 0.028 Cc	0.185 ± 0.055 Cc
Total chlorophyll (mg/g fw)								
Control	4.015 ± 0.249 Aa	3.640 ± 0.203 ABa	3.501 ± 0.453 Aa	2.412 ± 0.228 Bb	2.533 ± 0.110 Cb	2.924 ± 0.199 Ab	2.784 ± 0.123 Ab	2.518 ± 0.096 Ab
20	4.015 ± 0.249 Aa	3.605 ± 0.254 ABb	3.203 ± 0.051 Ac	2.501 ± 0.132 Bd	2.557 ± 0.065 Cd	2.061 ± 0.184 Be	1.585 ± 0.112 Cf	1.892 ± 0.109 Bef
60	4.015 ± 0.249 Aa	3.177 ± 0.086 Bb	3.347 ± 0.029 Ab	3.322 ± 0.123 Ab	3.216 ± 0.278 Ab	1.686 ± 0.095 Cc	1.874 ± 0.200 Bc	0.827 ± 0.126 Cd
100	4.015 ± 0.249 Aa	3.838 ± 0.204 Aa	2.474± 0.255 Bb	2.154 ± 0.214 Bb	2.046 ± 0.252 Bbc	1.597± 0.081 Ccd	1.466± 0.109 C d	0.953 ± 0.174 Ce
Carotenoids (mg/g fw)								
Control	0.523 ± 0.029 Aa	0.495 ± 0.027 Aab	0.441± 0.048 Abc	0.349 ± 0.027 Bde	0.304 ± 0.015 Be	0.383 ± 0.019 Acd	0.348 ± 0.006 Ade	0.319 ± 0.018 Ade
20	0.523 ± 0.029 Aa	0.477 ± 0.034 ABab	0.421± 0.008 Abc	0.336 ± 0.016 Bcd	0.320 ± 0.014 Bde	0.206 ± 0.099 ABf	0.218 ± 0.013 Cf	0.229 ± 0.017 Bef
60	0.523 ± 0.029 Aa	0.427 ± 0.013 Ba	0.416± 0.006 Aa	0.398 ± 0.020 Aab	0.406 ± 0.025 Aab	0.160 ± 0.130 Bc	0.283 ± 0.026 Bb	0.129 ± 0.018 Cc
100	0.523 ± 0.029 Aa	0.474 ± 0.018 ABa	0.316± 0.028 Bb	0.279 ± 0.032 Cbc	0.289 ± 0.035 Bbc	0.252 ± 0.012 ABbc	0.241 ± 0.019 Cc	0.141 ± 0.026 Cd
Chl-*a*/Chl-*b*								
Control	2.417 ± 0.102 Ad	2.643 ± 0.017Acd	2.635± 0.103 Bcd	2.705 ± 0.056 Bbc	2.615 ± 0.042 Bcd	2.928 ± 0.169 Ab	3.213 ± 0.129 Ba	3.444 ± 0.084 ABa
20	2.417 ± 0.102 Ab	2.682 ± 0.072 Ab	2.829± 0.038 Ab	2.878 ± 0.014 Ab	2.637 ± 0.096 Bb	2.916 ± 1.092 Ab	3.937 ± 0.135 Aa	3.241 ± 0.169 Bab
60	2.417 ± 0.102 Ab	2.756 ± 0.063 Ab	2.751± 0.022 ABb	2.511 ± 0.042 Cb	2.832 ± 0.094 Ab	2.857 ± 1.287 Ab	3.306 ± 0.158 Bab	4.148 ± 0.409 Aa
100	2.417 ± 0.102 Ac	2.697 ± 0.086 Ac	2.761± 0.036 ABc	2.684 ± 0.039 Bc	2.606 ± 0.038 Bc	3.658 ± 0.042 Ab	3.479 ± 0.086 Bb	4.251 ± 0.572 Aa

Mean values of all investigated parameters are presented ± SD ($n = 3$). Different capital letters in the same row indicate significant differences ($P < 0.05$) between the control and treatment groups; different lowercase letters in the same line indicate significant differences ($P < 0.01$) between the control and treatment group.

100 μmol/L Cd concentrations after 336 hr. The decrease in the pigment levels for the 20 μmol/L Cd concentration was greater than in the control group but lower than for the 60 or 100 μmol/L Cd concentrations. Furthermore, the decrease in the levels for 60 and 100 μmol/L Cd concentrations showed no significant differences at 336 hr. The inhibition effect of Cd on pigment production was enhanced by increasing the Cd concentration. However, the toxic effects towards *A. annua* from the 60 and 100 μmol/L Cd concentrations did not show significant differences at 336 hr.

Compared with the control group, Chl-*a* in the 20, 60, and 100 μmol/L Cd concentration treatment groups was decreased by 25.61%, 66.15%, and 60.51% after 336 hr respectively. These were each obviously lower than the control group levels at 144 and 12 hr. Chl-*b* levels for the 20, 60, and 100 μmol/L Cd concentrations were significantly lower than in the control group at 216 and 12 hr, and dropped by 21.05%, 71.93%, and 66.67% respectively compared with the control at 336 hr. Variations in total chlorophyll content for the 20, 60, and 100 μmol/L Cd concentrations were similar to the variations in Chl-*a*, and were dramatically lower than in the control at 144 and 12 hr. Moreover, these contents decreased by 25.00%, 67.06%, and 62.30% respectively compared with the control group at 336 hr. Compared with the control, the carotenoid levels for the 20, 60, and 100 μmol/L Cd concentrations decreased by 28.13%, 59.38%, and 56.25% respectively at 336 hr, dramatically and consistently lower than in the control group at 216, 144 and 12 hr.

Cd causes many physiological and biochemical changes in growing plants. Decrease in chlorophyll is the primary bioindicator of Cd phytotoxicity (Dinakar et al., 2008). A strong inhibition of chlorophyll biosynthesis by Cd has been found in maize (Parekh et al., 1990). This may be because Cd changes chlorophyll biosynthesis by inhibiting protochlorophyllide reductase, and also changes photosynthetic electron transport by inhibiting the water-splitting enzyme located on the oxidizing site of photosystem II (Van Assche and Clijsters, 1990).

We found that with increasing Cd concentration, the Chl-*a*, Chl-*b*, total chlorophyll, and carotenoid levels decreased. This also demonstrated the inhibition and toxicity of Cd for Chl-*a*, Chl-*b*, total chlorophyll, and carotenoids in *A. annua*.

The decrease in Chl-*b* content was greater than for Chl-*a*, carotenoids, or total chlorophyll for the same Cd concentration. In addition, compared with the control group, the decrease in Chl-*b* content in each treatment group was greater than for Chl-*a* or carotenoids at 336 hr. This indicated that Chl-*b* was more sensitive to Cd than were Chl-*a* or carotenoids. Hence, we found an increase in Chl-*a*/Chl-*b*. Khatun et al. (2008) also found that Chl-*b* was more affected by copper than was Chl-*a*.

Significant interactive effects between time and Cd concentrations on Chl-*a*, Chl-*b*, total chlorophyll, and carotenoids were seen (Table 2). The content of Chl-*a*, Chl-*b*, total chlorophyll, and carotenoids in plants treated with 100 μmol/L Cd concentrations were conspicuously lower than in the control group after 12 hr. With increasing Cd concentration, photosynthetic pigment levels decreased. This demonstrated that the toxic effect of Cd was enhanced by increasing Cd concentration. The pigment content at 336 hr was not significantly different for the 60 and 100 μmol/L concentrations, but the pigment content for the 100 μmol/L treatment exhibited an earlier drop, and then decreased more slowly than for the 60 μmol/L concentration. The toxicity of the 100 μmol/L Cd concentration treatment for photosynthetic pigments was clear during the initial time period. However, by activating the defense system for resisting the Cd stress, the decrease in photosynthetic pigments in the 100 μmol/L concentration was slowed in the later hours (Table 1).

Table 3 presents the effect of Cd on conductivity. Except for the control group, the conductivity for all Cd concentrations showed significant differences at 336 hr. For the treatment groups, the variation in conductivity increased at first and then decreased, and finally stabilized. For the 20 and 60 μmol/L Cd concentrations, conductivity clearly rose at first, to reach a maximum at 12 hr, and then fell to a minimum at 216 hr. For the 100 μmol/L Cd concentration, the maximum was at 4 hr, and the conductivity then significantly dropped to a minimum at 24 hr. All the treatment groups were slightly lower than the control group after 144 hr. The maximum value for the conductivity of each group rose with increasing Cd concentration.

At 4 hr, the conductivities for the 60 and 100 μmol/L Cd concentrations were greater than that of the control group or the 20 μmol/L Cd concentration. Furthermore, the greatest conductivity was observed for the 100 μmol/L concentration. At 12 hr, conductivities for the 20 and 60 μmol/L Cd concentrations were significantly greater than in the control group or the 100 μmol/L Cd concentration. Furthermore, the 60 μmol/L concentration exhibited the greatest conductivity. There were no conspicuous differ-

Table 2 Results of two-way ANOVA and Turkey multiple range tests for the effects of Cd concentrations and time on the Chl-*a*, Chl-*b*, total chlorophyll, carotenoids, Chl-*a*/Chl-*b*, conductivity, malondialdehyde (MDA), ascorbic acid (AsA), glutathione (GSH) and artemisinin content in *A. annua*

Source of variation	Chl-*a*	Chl-*b*	tChl	Carotenoids	Chl-*a*/Chl-*b*	Conductivity	MDA	AsA	GSH	Artemisinin
ANOVA *F*-values										
Time	229.908**	125.775**	229.023**	105.369**	23.182**	2.818**	4.240**	10.418**	4.726**	12.615**
Cd concentrations	66.078**	21.516**	55.808**	20.210**	2.134	0.404	2.564	4.277**	30.927**	1.181
Time×Cd concentrations	20.081**	5.978**	16.461**	6.400**	1.779*	2.986**	1.663	4.672**	2.441**	3.568**

The data are *F*-values for times, Cd concentrations and time×Cd concentrations interaction. * and ** indicate significant at $P < 0.05$ and 0.01, respectively.

Table 3 Effects of different Cd concentrations and treatment time on conductivity, malondialdehyde (MDA), ascorbic acid (AsA) and glutathione (GSH)

Cd conc.	Treatment time (hr)							
(μmol/L)	0	4	12	24	72	144	216	336
Conductivity (%)								
Control	2.509 ± 0.231 Aa	1.656 ± 0.173 Ca	1.751 ± 0.443 Ca	2.196 ± 0.820 Aa	1.840 ± 0.804 Aa	2.495±0.938 Aa	2.580 ± 0.550 Aa	2.254 ± 0.522 Aa
20	2.509 ± 0.231 Aa	1.311 ± 0.270 Cb	3.138 ± 0.881 Bb	2.863 ± 1.041 Aa	2.996 ± 0.647 Aa	1.574±0.243 Ab	1.156 ± 0.546 Ab	1.488 ± 0.564 Ab
60	2.509 ± 0.231 Aa	2.353 ± 0.332 Bb	3.732 ± 0.502 Aa	2.181 ± 0.646 Abc	1.984 ± 0.433 Abc	2.161±0.717 Abc	1.154 ± 0.168 Ac	1.686 ± 0.068 Abc
100	2.509 ± 0.231 Aa	4.445 ± 0.381 Aa	2.821 ± 0.435 Cc	0.955 ± 0.063 Ad	1.667 ± 0.263 Abd	1.876±0.702 Abd	1.245 ± 0.642 Ad	1.339 ± 0.396 Ad
MDA (μmol/g fw)								
Control	0.020 ± 0.002 Aabc	0.016 ± 0.001 Abc	0.020 ± 0.001 Aabc	0.019 ± 0.005 Aabc	0.024 ± 0.005 Aa	0.022±0.005 Aab	0.020 ± 0.002 Aabc	0.016 ± 0.002 Bc
20	0.020 ± 0.002 Ab	0.018 ± 0.002 Abc	0.017 ± 0.001 Bc	0.019 ± 0.002 Ab	0.023 ± 0.000 ABa	0.019±0.001 Ab	0.018 ± 0.001 ABbc	0.019 ± 0.001 Ab
60	0.020 ± 0.002 Aa	0.019 ± 0.005 Aa	0.016 ± 0.002 Ba	0.018 ± 0.000 Aa	0.020 ± 0.004 ABa	0.021±0.002 Aa	0.016 ± 0.002 ABa	0.018 ± 0.002 ABa
100	0.020 ± 0.002 Aa	0.020 ±0.004 Aa	0.017 ± 0.002 Bab	0.017 ± 0.001 Aab	0.017 ± 0.001 Bab	0.018±0.001 Aab	0.015 ± 0.002 Bb	0.019 ± 0.000 Aab
AsA (μmol/g fw)								
Control	1.276 ± 0.095 Ab	1.774 ± 0.583 Aa	1.664 ± 0.087 Aab	1.527 ± 0.027 Bab	1.649 ± 0.148 Aab	1.578±0.039 Aab	1.532 ± 0.097 Bab	1.658 ± 0.125 Bab
20	1.276 ± 0.095 Ac	2.022 ± 0.813 Ab	1.830 ± 0.361 Abc	1.560 ± 0.058 Bbc	1.531 ± 0.057 Abc	1.514±0.002 Bbc	1.564 ± 0.068 Bbc	2.923 ± 0.297 Aa
60	1.276 ± 0.095 Ad	1.542 ± 0.035 Ac	1.607 ± 0.013 Abc	1.823 ± 0.079 Aa	1.506 ± 0.022 Ac	1.557±0.018 ABc	1.511 ± 0.087 Bc	1.726 ± 0.124 Bab
100	1.276 ± 0.095 Ac	1.547 ± 0.060 Abc	1.550 ± 0.038 Abc	1.596 ± 0.051 Bbc	1.620 ± 0.153 Abc	1.534±0.032 ABbc	2.406 ± 0.438 Aa	1.804 ± 0.285 Bb
GSH (μmol/g fw)								
Control	0.079 ± 0.004 Aa	0.067 ± 0.006 Bab	0.074 ± 0.006 Cab	0.048 ± 0.008 Cc	0.070 ± 0.008 BCab	0.073±0.007 Bab	0.064 ± 0.008 Bb	0.073 ± 0.006 Bab
20	0.079 ± 0.004 Ab	0.088 ± 0.004 Ab	0.086 ± 0.002 ABb	0.082 ± 0.004 Ab	0.084 ± 0.003 Ab	0.113±0.032 Aa	0.089 ± 0.010 Ab	0.085 ± 0.007 Ab
60	0.079 ± 0.004 Aa	0.073 ± 0.006 Ba	0.077 ± 0.006 BCa	0.072 ± 0.006 Bab	0.061 ± 0.007C b	0.077±0.004 Ba	0.068 ± 0.008 Bab	0.076 ± 0.006 ABa
100	0.079 ± 0.004 Abc	0.089 ± 0.004 Aa	0.088 ± 0.004 Aab	0.082 ± 0.002 Aabc	0.075 ± 0.006 ABc	0.083 ± 0.006 ABabc	0.080±0.005 AB bc	0.086 ± 0.005 Aab

Mean values of all investigated parameters are presented ± SD ($n = 3$). Different capital letters in the same row indicate significant differences ($P < 0.05$) between the control and treatment groups; different lowercase letters in the same line indicate significant differences ($P < 0.05$) between the control and treatment groups.

ences between the control and treatment groups after 24 hr. The conductivity for the treatment groups was significantly lower than the control group at 216 hr.

Table 3 shows the effect of Cd on MDA content; this was considered a general indicator of lipid peroxidation. For the control and treatment groups, the variation in MDA content for the same Cd concentration was nearly the same; it initially decreased and then increased. The maximum MDA contents for the control, 20, 60, and 100 μmol/L Cd concentrations were at 72, 72, 144 and 336 hr respectively.

Analysis of the MDA content for different Cd concentrations at the same time showed that there were dramatic differences in MDA content between the control and treatment groups at 12 hr. However, the MDA content for all the treatment groups showed no significant differences.

Heavy metal toxicity is considered to induce the production of reactive oxygen species and may result in damage to cellular constituents. Actually, Cd cannot participate in the biological redox reactions by reason of its low redox potential. However, there is evidence that Cd might act as an oxidation-related disturbance, including the disturbance of lipid peroxidation (Sandalio et al., 2001). Membrane lipids and proteins are considered reliable indicators of oxidative stress in plants (Halliwell and Gutteridge, 1985; Palma et al., 2002).

Variation in conductivity in the treatment groups was nearly the same for the same treatment time; this clearly increased initially, then decreased to the same level as the control group at 24 hr. Cd concentration had a positive impact on the speed of variation in relative conductivity. Furthermore, the maximum conductivity for each treatment group also increased when the Cd concentration increased. This indicated that the plant cell membranes were sensitive to Cd stress. *A. annua* growing vigorously displayed a powerful defensive system that was able to resist stress. The defense reaction rate and extent were raised by increasing the stress level. The MDA content in the treatment groups showed no significant differences from the control group except at 12 hr. By increasing the Cd concentration, the maximum MDA level was delayed. Meanwhile, the maximum MDA content diminished. Conductivity contrasted with MDA content. Our results indicate that lipid peroxidation may not increase cell membrane permeability. The conductivity in treatment groups was lower than in the control from 216 hr onwards. Conversely, MDA content in the treatment groups was greater than in the control group at 336 hr. The toxicity effect of Cd on *A. annua* was enhanced with increasing time.

Table 3 illustrates the effect of different Cd concentrations on AsA and GSH content as a function of time.

In the control group, the AsA content clearly increased at 4 hr, and then only slightly changed during the rest of the time. The AsA content for the 20 μmol/L Cd concentration increased at 4 and 336 hr, reaching a maximum at 336 hr. In the 60 μmol/L Cd concentration treatment group, the AsA content clearly increased at 4 hr, reaching a maximum value at 24 hr. The AsA content for the 100 μmol/L Cd concentration changed only slightly between 0 and 144 hr, reaching a maximum at 216 hr. The maximum AsA value in the control group was lower than the values for the 20, 60, and 100 μmol/L Cd concentrations.

Analysis of the variation in AsA content for the different Cd concentrations at the same time showed that AsA for the 20, 60, and 100 μmol/L Cd concentrations was dramatically higher than in the control group at 336, 24 and 216 hr respectively. However, the AsA content in the treatment group showed no significant difference from the

control group over most of the time.

Table 3 presents the effect of Cd on GSH content. In the control group, GSH content reached a minimum at 24 hr, and only changed slightly during the rest of the time. The GSH content for the 20 μmol/L Cd concentration varied slightly between 0 and 336 hr; however, the GSH content had a significant increase only at 144 hr. The GSH content for the 60 μmol/L Cd concentration changed only slightly at first, and then sharply decreased to a minimum at 72 hr. The maximum values for GSH content for the 20, 60, and 100 μmol/L Cd concentrations were at 144, 12 and 4 hr, respectively.

Analysis of the variation in GSH content for different Cd concentrations at the same time indicated that the GSH content in all or some of the treatment groups was significantly greater than in the control group over most of the experimental time. The GSH content for the 20 μmol/L Cd concentration conspicuously exceeded that in the control group after 4 hr. For the 60 μmol/L Cd concentration, the GSH content was markedly greater than in the control group at 24 hr, while it showed no significant differences from the control group at other times. For the 100 μmol/L Cd concentration, the GSH content was conspicuously greater than in the control group at 4, 12, 24 and 336 hr. Moreover, the content for the 100 μmol/L Cd concentration slightly exceeded that in the control group at other times.

AsA and GSH are important components of non-enzymatic antioxidants. AsA and GSH are the primary antioxidants in plants, and are involved in several redox reactions, including directly reacting with reactive oxygen species and joining in the AsA-GSH cycle to clean H_2O_2 (May et al., 1998).

The obvious interaction of time and Cd concentrations can be observed in the AsA and GSH content (Table 2). In summary, the AsA content in the treatment groups showed no significant difference at the same concentration and in comparison with the control group, over most of the experimental time. This might be due to the inhibition of key enzymes that could catalyze AsA-GSH circulation. Or perhaps the stress level of Cd in our experiment was not able to activate AsA-GSH circulation. We found that the GSH content in all or some of the treatment groups was clearly greater than in the control group, and the time taken to reach the maximum GSH value was increased by increasing Cd concentration. Our findings were similar to those of Horemans et al. (2007). Cd stress was enhanced by increasing Cd concentration. The GSH response seemed dependent on the intensity of the Cd stress. Our experiment supported the conclusion that GSH was the main substance used to resist Cd stress. In addition, according to Horemans et al. (2007), the GSH content for treatment with a 60 μmol/L Cd concentration decreased sharply at 72 hr; possibly due to the generation of a chelating peptide. In the opinion of Polle and Schützendübel (2002), GSH content in a Cd-tolerant plant can rise during Cd stress, and vice versa. Based on the changes in GSH content observed during the experiment, *A. annua* may have a strong tolerance for Cd stress.

Table 4 shows the effect of Cd on artemisinin. The variation in artemisinin content for the 20 and 100 μmol/L Cd concentrations was nearly the same; artemisinin initially increased and then decreased sharply starting at 144 hr. For the 60 μmol/L Cd concentration, the artemisinin content was obviously lower than in the control group at 12 hr. The artemisinin content for the 100 μmol/L Cd concentration reached a maximum at 12 hr, while the maximum for the 20 and 100 μmol/L Cd concentrations was at 144 hr.

The variation of artemisinin content for treatments with different Cd concentrations at the same time indicated that the artemisinin content for the 20 and 100 μmol/L Cd concentrations was slightly greater than in the control group during the first 12 hr. At 144 hr, the content for the 20 and 100 μmol/L Cd concentrations clearly exceeded those in the control group, while the content for the 60 μmol/L Cd concentration was only slightly greater than in the control group. At 216 hr, the content in the treatment groups was greater than in the control group, but only the content for the 100 μmol/L Cd concentration significantly exceeded that in the control group. At 336 hr, the content in the treatment groups was obviously lower than in the control group (Table 4).

In conclusion, Cd stress can stimulate the synthesis and accumulation of artemisinin. Stimulation was obvious during the early times, especially for the 20 and 100 μmol/L Cd concentrations. Most of the active ingredients in traditional Chinese medicine are secondary metabolites in plants. These are the products of plants adapting to the environment over evolutionary time. The known main functions of secondary metabolites are pest-resistance, disease-resistance and stress-resistance. Huang (2006) advanced the stress-effect theory of famous-region drug formation for traditional Chinese medicine; this pointed out that environmental stresses advance the accumulation of secondary metabolites. Heavy metal stress can affect plant physiological and biochemical characteristics, and

Table 4 Variation in artemisinin content (%) in leaves of *A. annua* seedlings grown in different Cd concentrations over time

Cd concentration (μmol/L)	Treatment time (hr)				
	0	12	144	216	336
Control	0.885 ± 0.021 Aa	0.892 ± 0.018 Aa	0.845 ± 0.061 Ba	0.473 ± 0.027 Bb	0.632 ± 0.034 Ac
20	0.885 ± 0.021 Ab	0.991 ± 0.171 Aab	1.113 ± 0.018 Aa	0.681 ± 0.121 ABc	0.472 ± 0.082 Bd
60	0.885 ± 0.021 Ab	0.707 ± 0.090 Bb	0.946 ± 0.123 Bb	0.701 ± 0.176 ABb	0.421 ± 0.046 Ba
100	0.885 ± 0.021 Ab	1.091 ± 0.001 Aa	1.079 ± 0.015 Aa	0.815 ± 0.021 Ac	0.373 ± 0.011 Bd

Mean values of all investigated parameters are presented ± SD ($n = 3$). Different capital letters in the same row indicate significant differences ($P < 0.05$) between the control and treatment groups; different lowercase letters in the same line indicate significant differences ($P < 0.05$) between the control and treatment groups.

can bring serious damage to plants, even death. Heavy metal stress is therefore a crucial environmental stress. In this research, the artemisinin content in the treated groups rose in the first period of treatment time, and then went down at 336 hr. The increase in artemisinin content could be due to a high conversion rate of its immediate precursors viz. dihydroartemisinic acid to artemisinin (Wallaart et al., 1999). Artemisinic acid was found to be converted into arteannuin B by exposure to H_2O_2 by methylene blue-sensitized photo-oxygenation and into artemisinin by photooxidation, suggesting that conversion is brought by oxygen radicals (Irfan Qureshi et al., 2005). We conclude that artemisinin, as part of the stress-resistance system, can respond to Cd stress, as does the antioxidant defense system. Changes in the accumulation of secondary metabolites should have some relationship with the antioxidant system for the reason that both can respond to oxygen radicals. The artemisinin levels in the treatment groups were significantly lower than in the control group, at 336 hr. This might due to an enhanced toxic effect of Cd on *A. annua*. Long-time Cd treatment can cause damage to the cells, which could decrease the synthesis of artemisinin.

3 Conclusions

In sum, Cd treatment of growing seedlings of *Artemesia annua* impaired the production of photosynthetic pigments. Following the responses of conductivity and MDA, the main reason for changes in cell membrane permeability in *A. annua* need further study. Variation in AsA and GSH indicated that *A. annua* may have a strong tolerance towards Cd. The secondary metabolite, artemisinin, can resist Cd stress, as does the antioxidant system. An appropriate amount of Cd can promote the synthesis and accumulation of artemisinin. Thus we are able to increase the production of secondary metabolites by creating a reasonable stress over an appropriate time period. Further investigations are required to elucidate the molecular mechanisms of the antioxidant system and secondary metabolites against Cd stress.

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