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EFFECTS OF EXOGENOUS CALCIUM ON THE GROWTH AND PHYSIOLOGICAL TRAITS OF GARLIC SEEDLINGS UNDER CADMIUM STRESS

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ABSTRACT

In this study, the alleviation effect of exogenous calcium (Ca) against cadmium (Cd) toxicity to growth, photosynthetic characteristics, antioxidant properties, and absorption of nutrients was explored in nutrient solution-cultured garlic seedlings. The results indicated that Cd significantly inhibited the growth of garlic seedlings as well as reduced the chlorophyll content, net photosynthetic rate (P_n), transpiration rate (T_r), and stomatal conductance (G_s) of the garlic leaves. Cd also induced a peroxidation of garlic leaf cells due to an increased malondialdehyde (MDA) content; Cd also reduced the activities of the superoxide dismutase (SOD), peroxidase (POD), and catalase (CAT) in the garlic leaves. Additionally, Cd inhibited the absorption of nutrient elements (N, P, K, Ca, and Mg) but increased the Cd accumulation in garlic. Different concentrations of exogenous Ca alleviated the Cd toxicity. The morphological indexes (plant height, pseudostem diameter, pseudostem length and plant fresh weight) increased at low concentrations but decreased at high concentrations of exogenous Ca. From among the various exogenous Ca concentrations, the most effective dosage was either 2 or 3 mmol/L. The chlorophyll content and photosynthetic parameters (P_n, T_r, and G_s) of the leaves showed a similar tendency to the morphological indexes and were greatest when the exogenous Ca was 2 or 3 mmol/L. Moreover, exogenous Ca enhanced the SOD, POD and CAT activities and reduced the MDA content in the leaves, improved nutrient uptake, and reduced Cd accumulation, 2 or 3 mmol/L exogenous Ca was determined the most effective dose.

Key words: Cadmium, Calcium, Garlic, Growth, Physiological trait.

INTRODUCTION

For a variety of reasons, including industrial emissions, city refuse, sewage sludge and the application of phosphate fertilizers containing cadmium (Cd), the Cd levels in soils are increasing (McLaughlin *et al*., 1999; Adams *et al*., 2004; Ranieri *et al*., 2005). Cd is easily absorbed by plant roots and accumulates in different plant tissues, which poses a serious environmental threat when the accumulation reaches a toxic level (Sgherri *et al*., 2002), and it initially affects the roots of a plant since by more heavy metal ions are accumulated in roots than in shoots (EL-Beltagi and Mohamed, 2013). Moreover, It has been reported that Cd seriously impacts cell division and various metabolic activities in crops, thereby causing the chlorosis of organs, growth inhibition, and other damages, which ultimately results in a decline in both yield and quality (Bisova *et al*., 2004; Unyayar *et al*., 2006). Researchers have studied the toxic effects of Cd on different plants including the general symptoms of Cd toxicity, such as the inhibition of both growth and photosynthesis; the reduction of chlorophyll, sugar, and protein contents; and the decrease of related enzymes activities (EL-Beltagi and Mohamed, 2013; Farzadfar *et al*., 2013; Choong *et al*., 2014). In addition, Cd can interfere with the uptake of nutrient elements by affecting the permeability of plasma membranes, thus leading to a nutrient imbalance (Zhang *et al*., 2002). Furthermore, it is a growing concern that Cd has high bio-availability and can accumulate in agricultural products, thereby threatening human health through the food chain (Ci *et al*., 2009; Zhang *et al*., 2002).

Although there is no definitive explanation of how plants respond to Cd internally, increasing evidence indicates that the main toxicity of Cd may be associated with oxidative damage caused by reactive oxygen species (ROS) (EL-Beltagi and Mohamed, 2013; Radetski *et al*., 2004). While ROS generally stay at an acceptable level under the defense of antioxidant systems and therefore do not cause oxidative damage (Verma and Dubey, 2003), the ROS level can be greatly increased under stress conditions. For instance, stress conditions, such as heat, drought, salt, and heavy metals, can increase ROS generation in plants, and then, oxidative stress can result from this disturbance in the balance between ROS generation and removal (Cho and Seo, 2005). Moreover, the Cd-induced ROS can be scavenged by anti-oxidative enzymes such as superoxide dismutase (SOD), catalase (CAT), and peroxidase (POD) (Wu *et al*., 2003); however, Cd can inhibit the activities of these anti-oxidative enzymes and increase the malondialdehyde (MDA) content in plant cells (Maksymiec and Krupa, 2005; Lin *et al*., 2007; Ci *et al*., 2009).

For most plants, calcium (Ca) is not only a necessary element but is also a central regulator of plant growth and development, thereby playing a crucial role in the adaption to stress environments (EL-Beltagi and Mohamed, 2013; Hepler, 2005). Ca^{2+} can compete with Cd^{2+} for absorption by plant roots, and adding Ca^{2+} to the growth medium can reduce the damage caused to plants by excess Cd (Farzadfar *et al*., 2013; Suzuki, 2005). As such, exploring the alleviation effect of exogenous Ca poses an interesting area of research in combating Cd toxicity.

Presently, Cd is present in soils around the globe at varying levels, and these levels may increase and could, in the future, threaten human health through agricultural products. Furthermore, garlic (*Allium sativum* L.) is one of the *Allium* vegetables that is cultivated worldwide and has both high nutritional and medicinal value. Therefore, the purpose of this study was to explore the negative effects of Cd on the growth and physiological traits of garlic seedlings as well as to study the alleviation effect of exogenous Ca. In this study, we attempted to scientifically determine an appropriate dosage of exogenous Ca, to provide a reference for the Cd tolerance of garlic, and reduce Cd pollution in garlic products.

MATERIALS AND METHODS

Plant Culture and Treatments: The experiment was performed on garlic (*Allium sativum*, cultivar Jinxiang 3) in the glasshouse of Shandong Agricultural University, China, from October 2013 to May 2014. On October 15, healthy and equal-sized garlic cloves were chosen from bulbs that had not sprouted and were seeded in plastic pots containing foam boards in order to keep them in place. Each treatment included 20 pots with 12 garlic cloves per pot. The basic nutrient solution used in this experiment was a modified Hoagland and Arnon formulation, and all of the chemicals used were analytical grade. The composition of the nutrient solution was (mg/L): 210 N, 31 P, 234 K, 120 Ca, 48 Mg, 64 S, 2.8 Fe, 0.5 Mn, 0.5 B, 0.02 Cu, 0.05 Zn, and 0.01 Mo.

Different treatments were initiated after supplying distilled water for 1 month so as to promote rooting of the garlic cloves. The treatments were: (i) control (CK), nutrient solution alone; (ii) Cd stress (CK+Cd), 50 μmol/L Cd^{2+} added to CK; (iii) Cd plus exogenous Ca treatment 1 (CK+Cd+Ca1), 50 μmol/L Cd^{2+} and 1 mmol/L exogenous Ca^{2+} added to CK; (iv) Cd plus exogenous Ca treatment 2 (CK+Cd+Ca2), 50 μmol/L Cd^{2+} and 2 mmol/L exogenous Ca^{2+} added to CK; (v) Cd plus exogenous Ca treatment 3 (CK+Cd+Ca3), 50 μmol/L Cd^{2+} and 3 mmol/L exogenous Ca^{2+} added to CK; and (vi) Cd plus exogenous Ca treatment 4 (CK+Cd+Ca4), 50 μmol/L Cd^{2+} and 4 mmol/L exogenous Ca^{2+} added to CK. The pH of the nutrient solution was adjusted daily to 6.0 with a small amount of either diluted HCl or NaOH solution. The volume of the nutrient solution supplied to the plants' root zone was 60 L and was replaced every 3 days.

Sampling and Measuring: On March 3, the plants were harvested in order to determine the growth indexes (plant height, pseudostem diameter, pseudostem length, and plant fresh weight), the anti-oxidative enzymes (SOD, CAT, and POD) activities and MDA content of the leaves, and the elemental (Cd, N, P, K, Ca, Mg) contents of the roots. On March 15, both the photosynthetic parameters (net photosynthetic rate, transpiration rate, stomatal conductance, and intercellular CO_2 concentration) and the chlorophyll content were determined.

The chlorophyll content was determined according to the method of Arnon (1949) with some modification. The total N content was determined using the Kjeldahl method, the total K content was determined by flame photometry, and the total P content was measured by the Mo–Sb–Vc colorimetric method. The total Ca, Mg, and Cd contents were determined by an atomic absorption spectrophotometer.

The SOD activity was assayed by its ability to inhibit the photochemical reduction of nitroblue tetrazolium (NBT) (Farzadfar *et al*., 2013). The CAT activity was assayed according to the method of Farzadfar *et al*. (2013) with some modification. The POD activity was measured by the method of Zeng and Zhao (2013) with some modification. The lipid peroxidation level was expressed as the MDA content, which was determined from 2-thiobarbituric acid (TBA) reactive metabolites according to Zeng and Zhao (2013) with some modification.

Statistical Methods: All data presented are means of three replicates. The statistical analysis was performed using the SPSS statistical package version 18.0. Comparisons among the treatments were evaluated by one-way ANOVA using the least significant difference (LSD) test. Differences between the treatments were considered significant at $P < 0.05$.

RESULTS

Effect of Cd and Exogenous Ca on the Growth of Garlic Seedlings: Adding Cd (CK+Cd) caused a significant reduction in the garlic seedlings growth (Table 1). Moreover, the plant height, pseudostem diameter, pseudostem length, and plant fresh weight were each decreased by 39.4%, 34.1%, 38.3%, and 53.8%, respectively, as compared with the control (CK).

All of the exogenous Ca treatments improved the growth parameters of the garlic seedlings that were suffering from Cd exposure; however, the parameters remained lower than those of the control (CK) (Table 1).

Initially, the plant height, pseudostem diameter, and plant fresh weight increased, but as the exogenous Ca was increased, the parameters later decreased. Furthermore, the plant height, pseudostem diameter, and plant fresh weight achieved their maximum values at 2 mmol/L exogenous Ca (CK+Cd+Ca2) with respective increases of 54.4%, 26.0%, and 61.4% as compared to the Cd stress treatment (CK+Cd). In addition, the pseudostem length showed a similar tendency to the above indicators; it was greatest when the exogenous Ca was 3 mmol/L (CK+Cd+Ca3) and was enhanced by 45.2% as compared to the Cd stress treatment (CK+Cd). These results indicated that exogenous Ca could relieve the Cd-caused growth inhibition of garlic seedlings; however, excessive amounts produce a negative effect.

Table 1. Effects of Cd^{2+} (50 µmol/L) and exogenous Ca^{2+} on the plant height, pseudostem diameter, pseudostem length, and plant fresh weight of garlic seedlings.

Treatments	Plant height (cm)	Pseudostem diameter (mm)	Pseudostem length (cm)	Plant fresh weight (g)
CK	62.26a	19.47a	25.59a	157.68a
CK+Cd	37.70e	12.83e	15.78e	72.92e
CK+Cd+Ca1	42.93d	14.60d	19.54d	101.77c
CK+Cd+Ca2	58.20b	16.16b	21.08c	117.66b
CK+Cd+Ca3	55.16c	15.51bc	22.91b	99.64c
CK+Cd+Ca4	52.65c	15.03cd	20.47cd	85.26d

Mean values of three replicates followed by different letters are significantly different ($P < 0.05$). The same as below.

Effect of Cd and Exogenous Ca on the Chlorophyll Content of Garlic Leaves: The garlic leaves' chlorophyll content was seriously decreased by Cd (CK+Cd) and was reduced by 40.8% as compared to the control (CK) (Fig. 1). Furthermore, adding exogenous Ca to garlic seedlings under Cd stress (CK+Cd) increased the chlorophyll content, but the content remained less than that of the control (CK) (Fig. 1). The change tendency with increasing exogenous Ca concentration for the chlorophyll content was a unimodal curve, with a peak value at 2 mmol/L exogenous Ca (CK+Cd+Ca2); at which, it was enhanced by 37.8% as compared to the Cd stress treatment (CK+Cd). However, excessive amounts of exogenous Ca reduced the chlorophyll content of the garlic leaves.

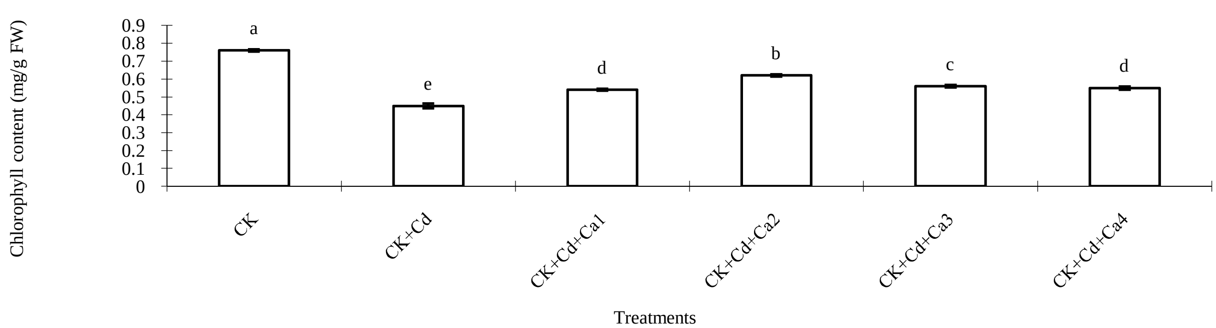

Fig. 1 Effects of Cd^{2+} (50 µmol/L) and exogenous Ca^{2+} on the chlorophyll content of garlic leaves. Each data point represents the mean ± S.D. of three replicates. Different letters indicate significant differences between the means ($P < 0.05$).

Effect of Cd and Exogenous Ca on the Photosynthetic Parameters of Garlic Leaves: The influence of Cd (CK+Cd) on the photosynthesis in the garlic leaves was highly extensive. More specifically, the net photosynthetic rate, transpiration rate, and stomatal conductance were each decreased to a great degree, whereas the intercellular CO_2 concentration was increased significantly (Table 2).

The exogenous Ca, however, relieved the inhibition of photosynthesis that was caused by Cd (Table 2). The net photosynthetic rate, transpiration rate, and stomatal conductance were improved to different extents, and the CK+Cd+Ca2 treatment produced the greatest effects. The intercellular CO_2 concentration continuously decreased along with increasing exogenous Ca concentration. These results demonstrate that exogenous Ca could effectively promote photosynthesis in Cd-affected garlic, but an excessive amount of exogenous Ca results in the opposite effect.

Table 2. Effects of Cd^{2+} (50 μmol/L) and exogenous Ca^{2+} on the photosynthetic parameters of garlic leaves.

Treatments	Net photosynthetic rate (μmol · m^{-2} · s^{-1})	Transpiration rate (mmol · m^{-2} · s^{-1})	Stomatal conductance (mmol · m^{-2} · s^{-1})	Intercellular CO_2 concentration (μmol/mol)
CK	9.73a	6.51a	357.33a	277.67d
CK+Cd	7.43c	4.20d	314.33d	338.67a
CK+Cd+Ca1	8.63b	4.75c	335.33bc	327.67ab
CK+Cd+Ca2	9.20ab	5.07b	348.67ab	315.33bc
CK+Cd+Ca3	9.03b	4.91bc	337.00bc	309.00c
CK+Cd+Ca4	8.73b	4.79c	329.00cd	303.67c

Effect of Cd and Exogenous Ca on the Activities of Anti-oxidative Enzymes and the MDA Content of Garlic Leaves: The activities of anti-oxidative enzymes (SOD, POD, and CAT) as well as the MDA content of the garlic leaves under Cd stress (CK+Cd) showed significant differences as compared to the control (CK) (Table 3). Specifically, the SOD, POD, and CAT activities were weakened by 26.0%, 21.4%, and 23.7%, respectively, whereas the MDA content was increased by 48.3%.

Furthermore, it was observed that exogenous Ca enhanced the SOD, POD, and CAT activities and reduced the MDA content of the garlic leaves exposed to Cd (Table 3). The exogenous Ca increased the SOD activity, and the best treatment was CK+Cd+Ca3, which enhanced the activity by 27.9% as compared to the Cd stress treatment (CK+Cd). Additionally, exogenous Ca also increased both the POD and CAT activities, and the best treatment was CK+Cd+Ca2, which enhanced the activities by 16.1% and 20.3%, respectively, as compared to the Cd stress treatment (CK+Cd). In contrast, the MDA content was decreased by the exogenous Ca and reached a minimum value at 2 mmol/L exogenous Ca (CK+Cd+Ca2) with a 19.0% reduction as compared to the Cd stress treatment (CK+Cd). In addition, higher levels of exogenous Ca did not produce superior results, as excessive Ca also caused a stress effect.

Table 3. Effects of Cd^{2+} (50 μmol/L) and exogenous Ca^{2+} on the SOD, POD, and CAT activities and the MDA content of garlic leaves.

Treatments	SOD activity (U/g FW)	POD activity (ΔOD_{470} · min^{-1} · g^{-1} FW)	CAT activity (ΔOD_{240} · min^{-1} · g^{-1} FW)	MDA content (nmol/g FW)
CK	172.05a	59.80a	25.86a	9.42d
CK+Cd	127.39d	47.02d	19.72c	13.97a
CK+Cd+Ca1	141.37cd	50.34cd	22.18bc	11.97bc
CK+Cd+Ca2	153.01bc	54.57b	23.73ab	11.32cd
CK+Cd+Ca3	162.89ab	51.14bc	22.25bc	12.30abc
CK+Cd+Ca4	158.10ab	47.36cd	20.57bc	13.42ab

Effect of Cd and Exogenous Ca on the Elemental Content of Garlic Roots: Cd inhibited the absorption of nutrient elements, whereas the accumulation of Cd increased (Table 4). As a result of the Cd stress, the N, P, K, Ca, and Mg contents of the roots were reduced by 19.1%, 38.3%, 34.0%, 55.2%, and 50.2%, respectively, as compared to the control (CK); the Cd content, however, increased by 280.8%.

In addition, it was found that exogenous Ca promoted the absorption of nutrient elements and decreased the Cd accumulation (Table 4). The tendency of the N, P, K, and Mg contents in the roots with increasing exogenous Ca concentration was a unimodal curve. Both the N and K contents reached their peak values at 2 mmol/L exogenous Ca (CK+Cd+Ca2); at which, they were increased by 15.5% and 25.8%, respectively, as compared to the Cd stress treatment

(CK+Cd). The P and Mg contents, however, reached their peak values at 3 mmol/L exogenous Ca (CK+Cd+Ca3) with respective increases of 37.4% and 62.3% as compared to the Cd stress treatment (CK+Cd). The Ca content of the roots was positively associated with the exogenous Ca concentration, while the Cd content of the roots decreased with increased Ca.

Table 4. Effects of Cd^{2+} (50 μmol/L) and exogenous Ca^{2+} on the Cd, N, P, K, Ca, and Mg contents of garlic roots.

Treatments	Cd (mg/kg DW)	N (mg/g DW)	P (mg/g DW)	K (mg/g DW)	Ca (mg/g DW)	Mg (mg/g DW)
CK	0.52e	151.83a	13.00a	47.15a	13.15a	6.13a
CK+Cd	1.98a	122.83d	8.02d	31.11e	5.89f	3.05d
CK+Cd+Ca1	1.90ab	130.78cd	9.44c	35.62c	7.24e	3.83c
CK+Cd+Ca2	1.87b	141.85b	10.34b	39.13b	8.63d	4.60b
CK+Cd+Ca3	1.76c	140.96b	11.02b	36.37c	10.23c	4.95b
CK+Cd+Ca4	1.63d	137.69bc	10.57b	32.87d	12.19b	4.65b

DISCUSSION

Cadmium is one of the most common heavy metal pollutants in soils worldwide and is capable of greatly limiting the productivity of crops because it is easily absorbed by plants and then accumulates in the plant tissues, where it interferes with essential physiological processes (Sgherri *et al*., 2002; Unyayar *et al*., 2006). The inhibition of growth is the most common symptom, which has been demonstrated by previous studies (Groppa *et al*., 2008; Daud *et al*., 2009; Farzadfar *et al*., 2013). In this study, 50 μmol/L Cd^{2+} seriously affected the morphological indexes of garlic seedlings and caused the seedlings to exhibit certain undesirable qualities, such as low plant height, pseudostem diameter, pseudostem length, and plant fresh weight. Furthermore, EL-Beltagi and Mohamed (2013) noted that Ca^{2+} has a similar ionic radius to Cd^{2+} and that they compete with each other for absorption by plants. In addition, Suzuki (2005) proved that the presence of Ca alleviated Cd-induced root growth inhibition. Here, exogenous Ca relieved Cd toxicity, and the morphological indexes were increased to a certain extent; however, they still remained lower than those of the control, which was similar to the results of Farzadfar *et al*. (2013). However, excessive exogenous Ca also inhibited the growth of garlic seedlings; the reason for which may be the ion toxicity of Ca. We found that an appropriate dosage of exogenous Ca was either 2 or 3 mmol/L.

Plant growth is inhibited as a consequence of reduced chlorophyll content and photosynthetic rate under heavy metal stress (Rodriguez-Serrano *et al*., 2009). This relationship can be directly applied to the detrimental effects of Cd toxicity as some studies have indicated that Cd causes iron deficiency and inhibits chlorophyll biosynthesis, which then results in a decline of photosynthetic rate (Wahid *et al*., 2008; Zulfqar *et al*., 2012). In this study, the chlorophyll content, photosynthetic rate (P_n), transpiration rate (T_r), and stomatal conductance (G_s) of garlic leaves were significantly decreased by Cd stress, which is in agreement with previous studies (Yu *et al*., 2013; Ci *et al*., 2009). However, exogenous Ca increased the chlorophyll content, P_n, T_r, and G_s of garlic leaves under Cd stress, and the best dosage was 2 mmol/L. Furthermore, both P_n and G_s declined under the Cd stress, whereas the intercellular CO_2 concentration (C_i) increased, which indicates that a non-stomatal limitation was the major reason for the inhibition of P_n. In contrast, a stomatal limitation was the major reason for the inhibition of P_n when the dosage of the exogenous Ca was too high because P_n, G_s, and C_i all declined (Farquhar and Sharkey, 1982).

Furthermore, an excessive production of reactive oxygen species (ROS) has been found in several plants that suffer from Cd stress (Markovska *et al*., 2009; Chen *et al*., 2010). A direct consequence of excess ROS is lipid peroxidation, and the extent of the cell damage may be connected with the increased MDA content (EL-Beltagi and Mohamed, 2013). However, the ROS levels in plant tissues under stress conditions are kept in balance by a highly efficient antioxidant system (Kafi *et al*., 2011). In previous studies, the Cd-induced lipid peroxidation in plants inhibited the activities of anti-oxidative enzymes (Yu *et al*., 2013; Schutzendubel and Polle, 2002). The possible mechanisms of these perturbations include the induction of oxidative stress and the replacement of some elements, such as Fe, Zn, and Mn, which are the essential cofactors of many anti-oxidative enzymes (Lopez-Millan *et al*., 2008). Here, Cd reduced the SOD, POD, and CAT activities as well as increased the MDA content of the garlic leaves, which shows that Cd causes lipid peroxidation damage and is consistent with results produced by other studies (Ci *et al*., 2009; Hassan *et al*., 2005). Ca is a second messenger of plants and can combine with Calmodulin (CaM); this combination (Ca-CaM) is closely related to the oxidation resistance of plants (Gong and Li, 1995; Hepler, 2005). Here, exogenous Ca effectively relieved the damage caused by

Cd and enhanced the SOD, POD, and CAT activities and decreased the MDA content; an appropriate dosage was either 2 or 3 mmol/L. The reason for this alleviation effect may be that Ca acts as a regulator of Cd tolerance and reduces Cd accumulation, but excessive Ca also induces peroxidative damage.

In higher plants, heavy metals such as Cd are likely to be transported across membranes via nutrient transporters or channels that are not completely selective; therefore, Cd^{2+} can enter cells via a channel that transports Ca^{2+} (Lopez-Climent *et al*., 2014; Choong *et al*., 2014). Thus, Cd affects the absorption of other elements, but the mechanisms are complex, and the conclusions are not consistent (Zhang *et al*., 2002; Choong *et al*., 2014). In this experiment, Cd seriously inhibited the absorption of N, P, K, Ca, and Mg and increased Cd accumulation, thereby destroying the nutrient balance in the garlic tissues. In contrast, exogenous Ca effectively promoted the absorption of N, P, K, Ca, and Mg in garlic suffering from Cd toxicity as well as reduced the accumulation of Cd in the garlic tissues. However, excessive exogenous Ca did not contribute to this remission and actually inhibited the absorption of nutrient elements, 2 or 3 mmol/L exogenous Ca produced the optimal results.

In conclusion, Cd^{2+} at 50 μmol/L caused serious damage to the garlic seedlings, which manifested in a detrimental influence on the growth, photosynthetic characteristics, antioxidant properties, and absorption of nutrients. Exogenous Ca^{2+} eased the toxic effects and improved the Cd tolerance of the garlic seedlings; the optimal dosage for these results was 2 or 3 mmol/L.

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