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Physiological responses of *Allium cepa* var. *agrogarum* L. plants to Cadmium stress

Respuestas fisiológicas de plantas de *Allium cepa* var. *agrogarum* L. al estrés por cadmio

Wang QL, DH Liu, JY Yue

Abstract. This study aimed to determine the effects of different Cd concentrations (0, 1, 10, 100, and 300 μM $CdCl_2$) on *Allium* plantlets. To achieve this goal, we examined the (1) dynamics of Cd^{2+} flux in the rhizosphere, (2) partitioning of Cd between roots and leaves, (3) formation of reactive oxygen species (ROS), (4) levels of H_2O_2 and chlorophyll, and (5) translocation of several macro- and micronutrients. A strong net Cd^{2+} influx was observed in the root apex after exposure to 100 μM Cd for 24 h. Exposure to either 1 or 10 μM Cd for 96 h presented no evident influence on root growth. However, treatments with 100 to 300 μM Cd for 72 h significantly inhibited root growth. ROS levels increased in roots and leaves with increasing Cd concentrations. The concentration of photosynthetic pigments, except for carotenoids on day 4, increased with increasing Cd concentrations and treatment duration. Cd accumulation decreased photosynthetic carbon assimilation but exerted no effects on diurnal patterns. Cd accumulated in roots and leaves, but a larger content was detected on roots than on leaves. Several macro- and micronutrients showed tissue- and concentration-specific responses to Cd.

Keywords: Cadmium (Cd) toxicity; Net cadmium flux; ICP; Oxidative stress; Photosynthesis.

Abbreviations: Cd: Cadmium; NRAMP: natural resistance-associated macrophage proteins; ICP-AES: inductively coupled plasma-atomic emission spectrometry analysis; LSD: the least significant difference; PSII: photosystem II; NMT: Non-invasive Micro-test Technique; ROS: reactive oxygen species.

Resumen. El propósito de este estudio fue determinar los efectos de varias concentraciones de Cd (0, 1, 10, 100, y 300 μM $CdCl_2$ en plantas de *Allium cepa* var. *agrogarum* L. Para alcanzar esta meta examinamos (1) la dinámica del flujo de Cd^{2+} en la rizósfera, (2) el particionamiento de Cd entre las raíces y las hojas, (3) la formación de especies reactivas al oxígeno (ROS), (4) niveles de H_2O_2 y clorofila, y (5) la movilización de varios micro- y macronutrientes. Un elevado influjo de Cd^{2+} neto se observó en el ápice radical después de la exposición a 100 μM Cd por 24 h. La exposición a 1 o 10 μM Cd por 96 h no presentó una influencia evidente en el crecimiento radical. Sin embargo, los tratamientos con 100 a 300 μM Cd por 72 h inhibieron significativamente el crecimiento radical. Los niveles de ROS se incrementaron en hojas y raíces con aumentos en las concentraciones de Cd. La concentración de pigmentos fotosintéticos, excepto los carotenoides el día 4, se incrementó con aumentos en las concentraciones de Cd y duración del tratamiento. La acumulación de Cd disminuyó la asimilación de C fotosintético pero no tuvo efectos en los modelos diurnos. El Cd se acumuló en las raíces y en las hojas, pero se detectó un mayor contenido en las raíces que en las hojas. Varios macro- y micronutrientes mostraron respuestas específicas del tejido y a la concentración de Cd.

Palabras clave: Toxicidad del Cadmio (Cd); flujo neto de cadmio; ICP; Estrés oxidativo; Fotosíntesis.

Abbreviations: Cd: Cadmio; NRAMP: Proteínas de macrófagos asociadas con resistencia natural; ICP-AES: análisis de espectrometría de emisión atómica-plasma asociada inductivamente; LSD: diferencia mínima significativa; PSII: Fotosistema II; NMT: Técnica de micro-prueba no invasiva; ROS: especies reactivas al oxígeno.

Tianjin Key Laboratory of Animal and Plant Resistance, Tianjin Normal University, Tianjin, 300387, P.R. China.
Address correspondence to: Jieyu Yue, Tel: 86-22-2376 6823, Fax: 86-22-2376 6359, *e-mail:* skyyjy@mail.tjnu.edu.cn
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INTRODUCTION

Heavy metal (e.g., Cd) contamination due to anthropogenic activities, such as mining, urban traffic, burning of fossil fuels, and phosphate fertilizer production, is a serious environmental problem worldwide (McLaughlin et al., 2000; Zhang & Wong, 2007; Ikenaka et al., 2010; Liu et al., 2011). Cd is nonessential but biologically toxic; particularly, high Cd concentrations in the soil are phytotoxic (Schutzendubel & Polle, 2002; He et al., 2011; Rascio & Navari-Izzo, 2011). The capacity of this metal to enter the cell through the existing mineral uptake machinery also constitutes a serious threat to human health (Peralta et al., 2009; Straif et al., 2009; Lin & Aarts, 2012). Cd entry to root cells is the first key process for phytoremediation, but only a few reports have described the dynamics of Cd^{2+} flux along the roots in monocots by using ion-selective microelectrodes (He et al., 2011). In particular, insufficient information is available about the dynamics of Cd^{2+} flux in the rhizosphere of *Allium* plants.

Cd exposure inhibits plant growth by reducing mitotic activity, inducing chromosomal aberrations, and causing toxicity to nucleoli in the apical meristem (Liu et al., 2003/2004; Zhang et al., 2009; Qin et al., 2010). Cd also disturbs plant physiology and metabolism of plants by altering Chlorophyll *a* and *b* (Chl *a* and *b*) contents (Mobin & Khan 2007; He et al., 2011); reducing net photosynthetic rate, stomatal conductance, and leaf transpiration (Souza et al., 2011); and damaging macromolecules, which mainly include proteins, lipids (Skorzynska-Polit & Krupa 2006), and DNA (Li et al., 2005; Cambier et al., 2010). Moreover, Cd induces mitochondrial damage and triggers cell death through apoptosis or necrosis (Thijssen et al., 2007). However, most studies on Cd toxicity and detoxification mechanisms focused on Cd-hyperaccumulating plants that have developed different mechanisms to cope with Cd; such plants include *Thlapsi caerulescens* (Lombi et al., 2002), *Arabidopsis halleri* (Bert et al., 2002; Weber et al., 2006; Zhao et al., 2006; Gallego et al., 2012), *Thlaspi praecox*, and *Sedum alfredii* (Van de Mortel et al., 2008). These studies provided valuable insights into the metal homeostasis mechanisms of plants to regulate the cellular concentrations of metal ions (Gallego et al., 2012). However, evidence regarding the mechanisms by which metal-sensitive species such as *Allium cepa* reduce the negative consequences of metal toxicity is insufficient. Some studies gathered information on the coping mechanisms of *A. cepa* with heavy metals (Liu et al., 1995; Qin et al., 2010), but data on the response of *A. cepa* var. *agrogarum* L. to Cd stress are limited.

As a useful biomarker for environmental monitoring, *A. cepa var. agrogarum* L. was selected to investigate the dynamics of Cd^{2+} flux in the rhizosphere and to explore the plant internal partitioning of Cd and other minerals (Ca, Mg, Fe, Mn, and Zn) between roots and leaves. This investigation was conducted in relation to energy metabolism; reactive oxygen species (ROS) formation; antioxidant, chlorophyll, and carotenoid concentrations; and photosynthetic characteristics. The findings of this study provided insights into the molecular mechanisms of *Allium* seedlings in response to Cd stress.

MATERIALS AND METHODS

Plant material, germination and cadmium treatment. Healthy and equal-size onion bulbs (*Allium cepa* var. *agrogarum* L.) were chosen. The bases of bulbs remained submerged in water to produce roots at 25 °C. When the roots reached about 1.0 cm in length, the germinated bulbs were transferred into the 1/4 Hoagland' nutrient solution which was exchanged at 1 day intervals. After roots were approximately 3 cm long, they were exposed to 0, 1, 10, 100 or 300 μM $CdCl_2$ concentrations in the nutrient solution. A parallel culture was grown without subcultivation at 25 °C as a positive control.

Macroscopic observations were made at the end of 4, 8 and 12 days. In each treatment, 5 plants were examined, and root and leaf lengths were measured every 4 days.

Measurement of net Cd^{2+} flux in roots. To monitor net Cd^{2+} flux in roots of *Allium cepa* var. *agrogarum* L. exposed to 100 μM $CdCl_2$, white fine roots from 3-day-old plants were selected. The net Cd^{2+} flux was measured non-invasively by using the Non-invasive Micro-test Technique (the NMT system BIO-IM; Younger USA, LLC, Amherst, MA) at the company (Xuyue Science & Technology Co., Ltd. Beijing, China). The NMT system and its application on ion flux detection were described in detail (Pineros et al., 1998; Farrell et al., 2005; Xu et al., 2006; Ma et al., 2010). Briefly, the ion-selective microelectrode with an external tip (ca. 2-4 μm in diameter, YoungerUSA) was manufactured and silanized with tributylchlorosilane, and the tip was backfilled with a commercially available ion-selective cocktail (Cadmium Ionophore I, 20909, Sigma-Aldrich, St Louis, MO). Prior to the net Cd^{2+} flux measurement, the microelectrode was calibrated in 50 and 500 μM Cd^{2+} and only electrodes with Nernstian slopes more than 25 mV per 10 times concentration difference were used. Three fine roots per plant (eight plants in total) were used for this analysis. The white fine roots excised from plants exposed to 100 μM $CdCl_2$ for 24 h were immediately transferred to a Petri dish containing 5 mL of a measuring solution (0.1 mM $CdCl_2$; 0.05 mM KCl, 0.25 mM NaCl, 0.15 mM MES and 0.1 mM Na_2SO_4, pH 6.0). To determine the appropriate point for measurement along the root tip, a preliminary experiment was carried out with an initial measurement at the root tip followed by 400 μm walk steps (Fig. 1A). Gradients of Cd^{2+} near to the root surface (ca. 2–5 μm) were measured by moving the Cd^{2+}-selective microelectrode between two positions (with a distance of 30 μm) in perpendicular direction to the root surface. The recording rate for Cd^{2+} flux was 10

readings per 64 s. The Cd^{2+} flux was recorded for a period of 4 min. Acquisition of root images and processing of Cd^{2+} flux data were performed with an IM-FLUX software attached to the NMT system.)

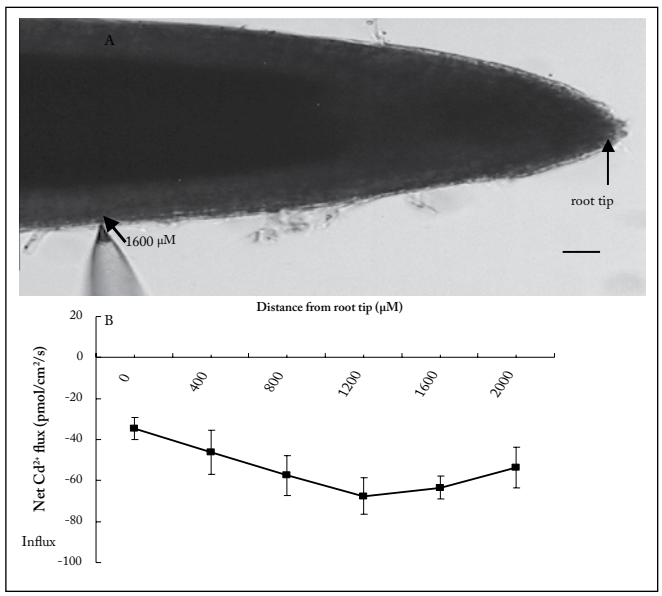

Fig. 1. Root tip (A) and net Cd^{2+} flux along the root tip (B) of *Allium cepa* var. *agrogarum* L. after exposure to 100 μM $CdCl_2$ for 24h (Bar=100 μm). Symbols are means ± SE (n=6). The negative values indicate Cd^{2+} influx. Net Cd^{2+} flux in roots was measured non-invasively by using NMT (for details see the text).

Fig. 1. Ápice radical (A) y flujo neto de Cd^{2+} a lo largo del ápice radical (B) de *Allium cepa* var. *agrogarum* L. después de la exposición a 100 μM $CdCl_2$ durante 24 h (Barra=100 μm). Los símbolos son promedios ± SE (n=6). Los valores negativos indican el influjo de Cd^{2+}. El flujo neto de Cd^{2+} en las raíces se midió en forma no invasiva usando NMT (ver el texto por detalles).

Determination of O_2^- and H_2O_2. We measured O_2^- content in plant materials using the aerated method following Lei et al. (2006). Samples (0.2 g) were ground in liquid nitrogen. The obtained powder was suspended in 1 mL of 50 mM potassium phosphate buffer (pH 7.8) and then centrifuged (10000 g, 4 °C, 20 min). A 1 mL aliquot of the supernatant was mixed with 1 mL of 1 mM hydroxylamine hydrochloride. Subsequently, the reaction mixture was incubated at 25 °C for 60 min prior to adding 1 mL of 17 mM *p*-aminobenzene sulfonic acid and 1 mL of 7 mM α-naphthylamine. After further incubation (25 °C, 20 min), the absorbance of the mixture was spectrophotometrically recorded at 530 nm.

The concentration of H_2O_2 in plant materials was analyzed as described by Lei et al. (2007). The obtained fine powder of fresh tissues (0.2 g) was extracted in 1 mL of acetocaustin and then centrifuged (10000 g, 4 °C, 20 min). The supernatant was discarded, and the pellet was dissolved in 3 mL of 2 M H_2SO_4. Absorbance was spectrophotometrically recorded at 415 nm.

Photosynthetic pigments analysis. To determine chlorophyll and carotenoid concentration on leaves, fine powder of fresh leaves (0.2 g) was extracted for 24 h in 8 mL of 80% acetone in darkness. The concentrations of chlorophyll *a*, chlorophyll *b* and carotenoids in the extracts were determined by a spectrophotometer (UV-2550, Shimadzu, Japan) at 663, 646 and 470 nm, respectively (Wellburn, 1994).

Measurement of photosynthesis parameters. All assessments of net photosynthetic rate were tested using a portable photosynthesis system (LI-6400; Licor, Lincoln, NB, USA) mounted with a red LED light source (6400-02B, Licor). The diurnal variations of photosynthesis parameters were measured as follows. On a cloudless, sunny day, the net photosynthetic rate, stomatal conductance, and transpiration rate were successively measured with an interval of 2 h in a diurnal course from 08:00 to 14:00 h. Photosynthetic active radiation, air temperature, leaf temperature, block temperature, relative humidity, CO_2 concentration in the air, and vapor pressure deficit were also automatically recorded. Measurements were obtained on the main functional leaf of the stem on five selected plants using three repeats for each study variable, respectively. The measurement of all study variables for one repeat was finished within 25 min.

Inductively coupled plasma-atomic emission spectrometry (ICP–AES). Seedlings were harvested after 12 d for ICP–AES. The contents of Cd, Ca, Mg, Mn, Fe, and Zn were determined after a prior mineralization step by using ICP–AES (LEEMAN LABS Inc., NH, U.S.A.) as described by Duan (2003). Samples were prepared in accordance with the procedure described by Khan et al. (2013) and Liu et al. (2008).

Statistical analysis. For each treatment, at least five plants were analyzed, and all experiments were repeated for at least five times. Results are presented as means ± SD. For statistical analysis, one-way ANOVA and t-test were used to determine the Least Significant Difference at $P<0.05$.

RESULTS

Net Cd^{2+} flux in roots. To detect the spatial dynamics of Cd^{2+} movement along the root tips of the *Allium* plants, the net Cd^{2+} flux was analyzed by a NMT (non-invasive microtest technology, NMT) (Fig. 1b) after Cd exposure for 24 h. At the root tips of *A. cepa* var. *agrogarum* L., the net Cd^{2+} flux displayed an influx ranging from 34.80 ± 5.36 pmol/cm²/s to 67.64 ± 8.93 pmol/cm²/s, depending on the distance from the root tips (Fig. 1A). The net influx of Cd^{2+} markedly decreased

at 800 μm from the root tips (Fig. 1B). To further analyze the temporal dynamics of Cd^{2+} flux, the average net Cd^{2+} influx at 800, 1200, and 1600 μm from the root tips was monitored. The Cd^{2+} influx at 800 μm to 1600 μm from the root tips varied from 57.43 ± 9.74 pmol/cm²/s to 67.64 ± 8.93 pmol/cm²/s, with the highest net influx recorded at 1200 μm from the root tips.

Macroscopic effects of Cd on root growth. The effects of Cd on the root growth of the *Allium* plants varied with Cd concentration and treatment time (Figs. 2 and 3). Treatment with >10 μM Cd inhibited *Allium* plant root growth, whereas that with <10 μM Cd exerted no negative effect on root growth. Roots showed a significantly improved growth ($P<0.05$) and normal morphology during the entire treatment with 1 μM Cd. By contrast, they displayed a significantly inhibited growth ($P<0.05$), and stunted and slightly bent tips, after 48 h of treatment with 100 μM Cd. After 24 h of treatment with 300 Cd μM, the roots either grew slowly or stopped growing ($P<0.05$), and the root tips were seriously stunted and bent in various directions.

Fig. 2. Effect of various concentrations of Cd on root growth of *Allium cepa* var. *agrogarum* L. (96 h).
Fig. 2. Efecto de varias concentraciones de Cd en el crecimiento radical de *Allium cepa* var. *agrogarum* L. (96 h).

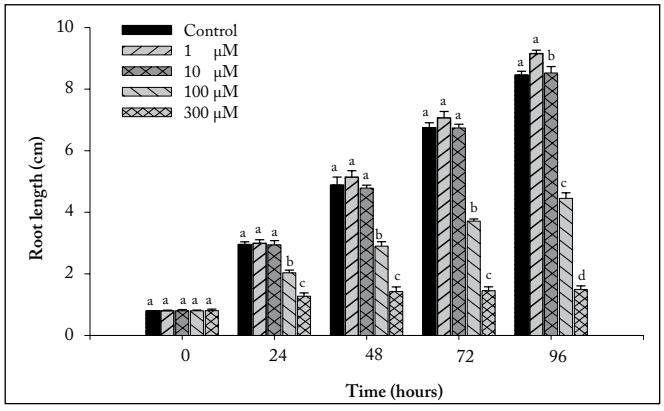

Fig. 3. Effect of various concentrations of Cd on root length of *Allium cepa* var. *agrogarum* L. at various exposure times. Within each exposure time, histograms with different letters are significantly different (n=15, $P<0.05$).
Fig. 3. Efecto de varias concentraciones de Cd en la longitud radical de *Allium cepa* var. *agrogarum* L. después de varios tiempos de exposición. Dentro de cada tiempo de exposición, los histogramas con diferentes letras son significativamente diferentes (n=15, $P<0,05$).

Effects of Cd on contents of O_2^- and H_2O_2. The O^{2-} contents in the roots and leaves of the *Allium* plants varied with Cd concentration and treatment duration. The O^{2-} content in roots exposed to 1 μM to 300 μM Cd significantly increased ($P<0.05$) as compared with that in the control (Fig. 4a). O^{2-} content increased when increasing treatment time, and then subsequently decreased within 4 d to 12 d of Cd treatment. In addition, the O^{2-} content in the roots induced by 300 μM Cd was nearly twice that in the control after 8 d. The O^{2-} content in the leaves was considerably higher than that in the roots (Figs. 4a and 4b). Figure 4b shows the effects of different Cd concentrations on the O^{2-} content of *A. cepa* var. *agrogarum* leaves. The O^{2-} level on leaves was significantly higher ($P<0.05$) than that in the control from 1 μM to 300 μM Cd after 8 d. After 4 d, the O^{2-} level on leaves was not significantly higher than that on roots ($P>0.05$). The activity of O^{2-} on leaves peaked after exposure to 300 μM Cd.

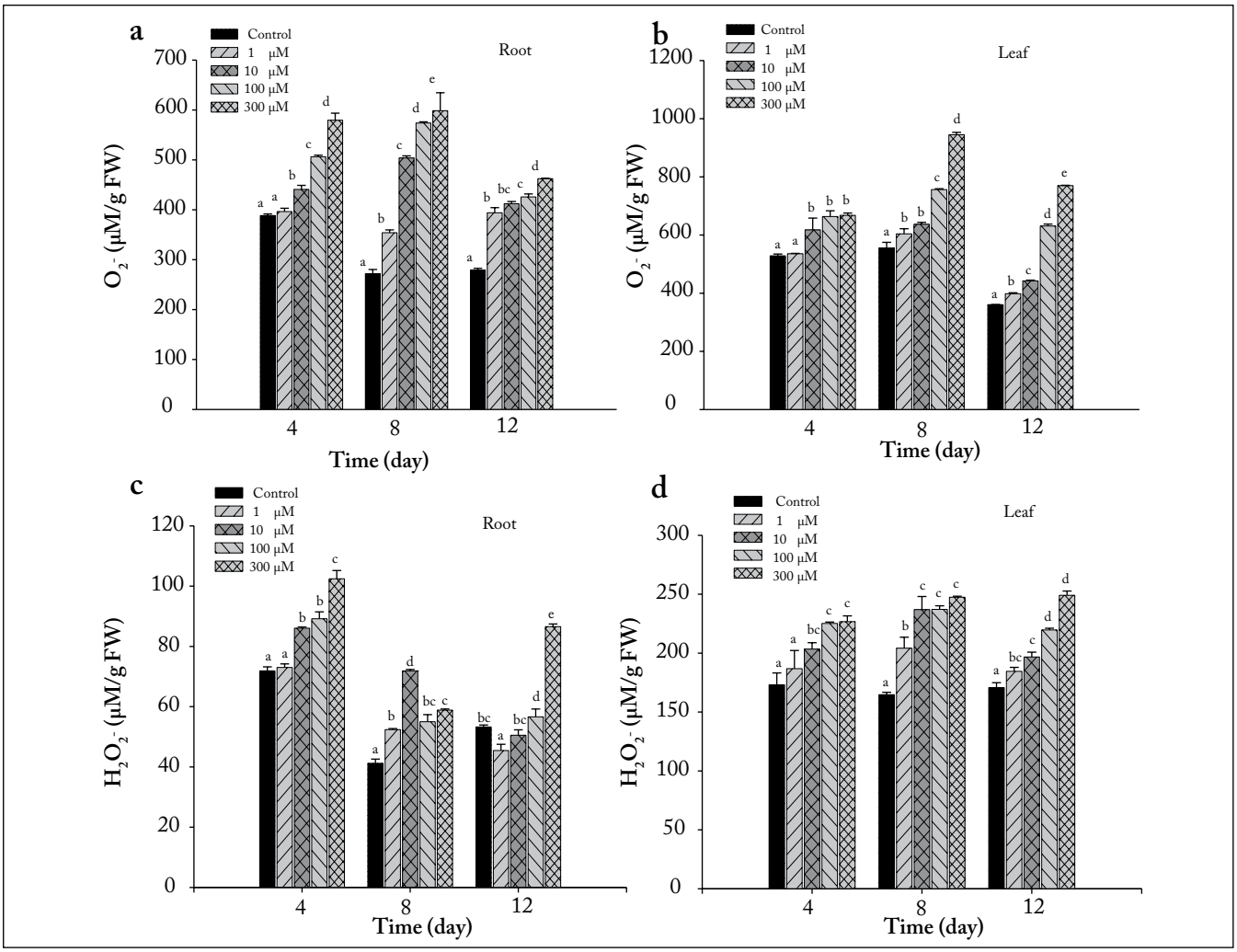

Fig. 4. Effect of different concentrations of Cd on the activities of O^{2-} and H_2O_2 in *Allium cepa* var. *agrogarum* L. exposed to Cd stress over 12 days. (a) O^{2-} in roots, (b) O^{2-} in leaves, (c) H_2O_2 in roots, (d) H_2O_2 in leaves. Histograms indicate means ± SE. Different letters on the histograms for the same tissue indicate significant differences among treatments (P<0.05, t-test).

Fig. 4. Efecto de diferentes concentraciones de Cd en las actividades del O^{2-} y H_2O_2 en *Allium cepa* var. *agrogarum* expuesta a estrés de Cd durante 12 días. (a) O^{2-} en las raíces, (b) O^{2-} en las hojas, (c) H_2O_2 en las raíces, (d) H_2O_2 en las hojas. Los histogramas indican promedios ± EE. Diferentes letras sobre los histogramas para el mismo tejido indican diferencias significativas entre los tratamientos (P<0,05; t-test).

The effects of different Cd concentrations on H_2O_2 activity are shown in Figures 4c and 4d. H_2O_2 activity showed no obvious consistent trend, and was lower on roots after 8 d of treatment. The changes in root H_2O_2 activity differed from those in root O^{2-} level and leaf H_2O_2 activity under Cd treatment (Fig. 4c). As shown in Fig. 4d, the H_2O_2 activity on leaves exposed to 1 μM and 10 μM Cd was significantly higher (P<0.05) that that in the control after 8 d. No evident differences in H_2O_2 activity on leaves were detected after treatment with 100 and 300 μM Cd during the entire treatment (Fig. 4d).

Effects of Cd on photosynthetic pigment concentrations. The photosynthetic pigment concentrations in the *Allium* plants varied with Cd concentration and treatment duration (Table 1). The photosynthetic pigment concentration in the leaves exposed to all Cd concentrations increased with treatment duration. The photosynthetic pigment concentrations in the group treated with 300 μM Cd were significantly higher (P<0.05) that those in the control and other treatment groups. Exposure from 1 μM to 300 μM Cd for 8 d significantly increased (P<0.05) the concentrations of Chl *a*, Chl *b*, and Chl *a* + *b* in the leaves as compared with those in the control and with each other. The concentrations of Chl *a*, Chl

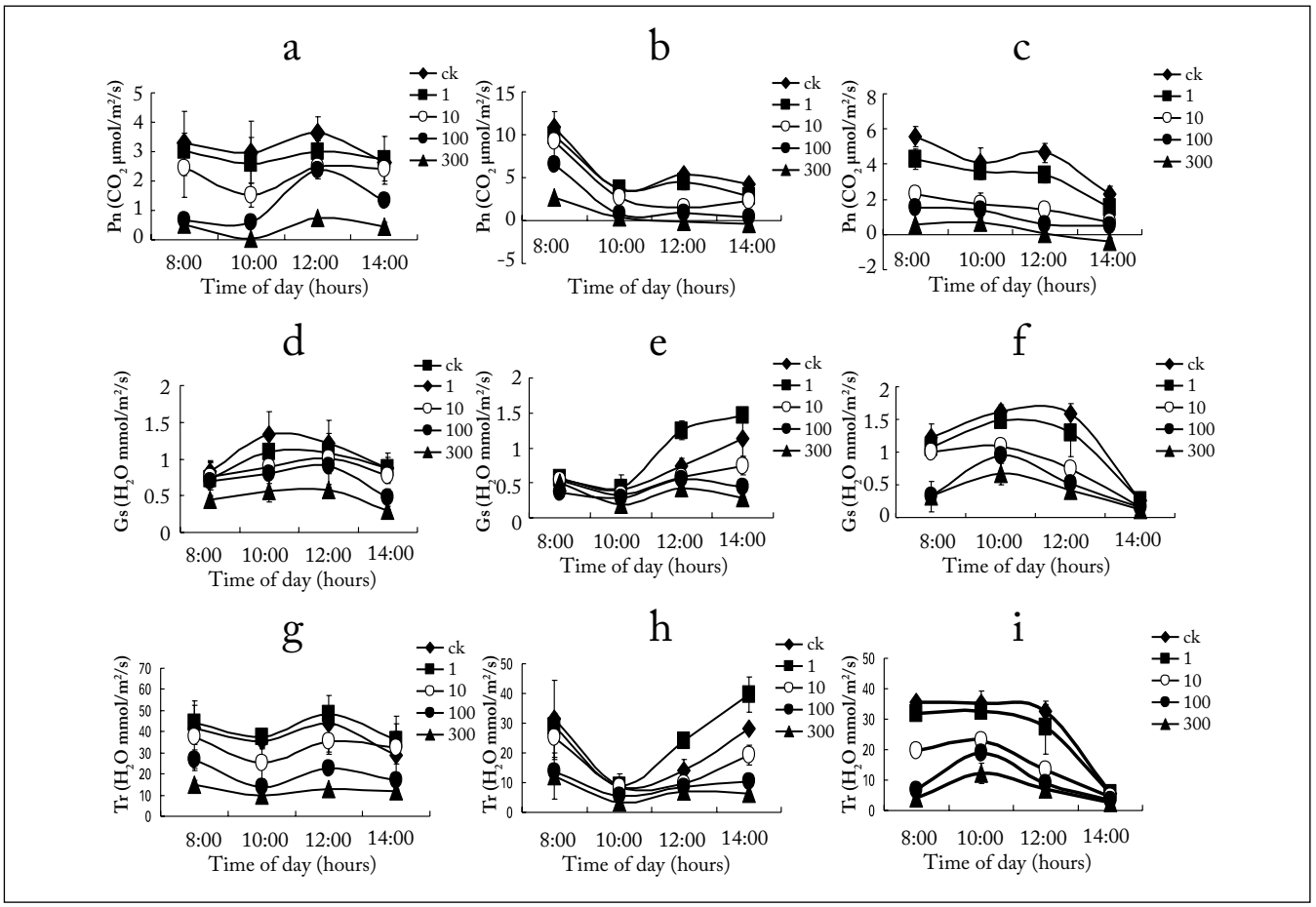

Fig. 5. Effect of different concentrations (μM) of Cd on diurnal variations of the net photosynthetic rate (Pn), stomatal conductance (Gs), and transpiration rate (Tr) in *Allium cepa* var. *agrogarum* L. exposed to Cd stress over 12 days. (a) Pn on 4th day, (b) Pn on 8th day, (c) Pn on 12th day, (d) Gs on 4th day, (e) Gs on 8th day, (f) Gs on 12th day, (g) Tr on 4th day, (h) Tr on 8th day, (i) Tr on 12th day. Data are the means of three replicates with standard errors shown by bars.

Fig. 5. Efecto de diferentes concentraciones (μM) de Cd en la variación diurna de la tasa fotosintética neta (Pn), conductancia estomática (Gs) y tasa de transpiración (Tr) en *Allium cepa* var. *agorgarum* L. expuesto a estrés por Cd durante 12 días. (a) Pn en el 4to día, (b) Pn en el 8vo día, (c) Pn en el 12do día, (d) Gs en el 4to día, (e) Gs en el 8vo día, (f) Gs en el 12do día, (g) Tr en el 4to día, (h) Tr en el 8vo día, (i) Tr en el 12do día. El error estándar se muestra como barras verticales.

b, and Chl *a* + *b* under exposure from 1 μM to 100 μM Cd showed no considerable changes in comparison with the control after 12 d . However, these concentrations peaked after treatment with 300 μM Cd for 12 d [Chl *a*, 0.719 ± 0.016 mg/g; Chl *b*, 0.178 ± 0.003 mg/g; Chl (*a* + *b*) 0.897 ± 0.013 mg/g]. The pigment concentrations on day 8 were 19.5%, 17.2%, 23.9%, 29.7%, and 42.8% higher than those on day 4 after exposure to 0, 1, 10, 100, and 300 μM Cd, respectively. These concentrations continued to increase with treatment duration; an exception to this result was the group treated with 300 μM Cd. The pigment concentrations on day 12 were 51.9%, 26.1%, 15.7%, 9.5%, and 14.9% higher than those on day 8.

Table 1 shows that carotenoid concentration increased with increasing Cd concentration from 1 μM to 10 μM for 4 d. The carotenoid concentrations peaked at 10 μM Cd and then decreased. The changes in carotenoid concentration after 8 to 12 d of treatment were similar to those in Chl *a* and Chl *b* concentrations.

Diurnal changes in photosynthetic characteristics

Variation in net photosynthetic rate. The net photosynthetic rate of *Allium* functional leaves varied with Cd concentration and treatment duration. This rate increased after 4 d to 12 d of treatment and thereafter it decreased. Bimodal and unimodal diurnal variation patterns of the net photosynthetic rate were observed on leaves. The *Allium* plants exhibited unimodal diurnal patterns from 8:00 to 14:00 h (Figs. 5a to 5c), and net photosynthetic rates of plants negatively correlated with treatment duration. In particular, the net photosynthetic rates of *Allium* plants increased at 08:00 h, gradually decreased with time, reached the minimum at 10:00 h, and then evidently

Table 1. Variation of photosynthetic pigment concentrations (mg/g fresh weight) on leaves of *Allium cepa* variety *agrogarum* L. which were exposed to various CdCl2 concentrations for 4, 8 or 12 days.
Tabla 1. Variación en la concentración de pigmentos fotosintéticos (mg/g peso fresco) en hojas de *Allium cepa* var. *agrogarum* L. que fueron expuestas a varias concentraciones de CdCl2 durante 4, 8 ó 12 días.

Time(d)	Cd(μM)	Chl*a*	Chl*b*	Chl*(a+b)*	Car
4	0	0.285 ± 0.004 a	0.060 ± 0.000 a	0.345 ± 0.004 a	0.076 ± 0.005 a
	1	0.358 ± 0.004 b	0.074 ± 0.002 b	0.433 ± 0.006 b	0.094 ± 0.001 cd
	10	0.391 ± 0.002 c	0.087 ± 0.001 c	0.478 ± 0.003 c	0.098 ± 0.001 d
	100	0.419 ± 0.001 d	0.095 ± 0.001 d	0.514 ± 0.001 d	0.090 ± 0.000 b
	300	0.438 ± 0.000 e	0.098 ± 0.002 e	0.536 ± 0.002 e	0.092 ± 0.001 b
8	0	0.341 ± 0.005 a	0.076 ± 0.003 a	0.417 ± 0.417 a	0.072 ± 0.004 a
	1	0.420 ± 0.020 b	0.098 ± 0.005 b	0.518 ± 0.518 b	0.081 ± 0.003 b
	10	0.485 ± 0.006 c	0.120 ± 0.006 c	0.605 ± 0.605 c	0.093 ± 0.002 c
	100	0.543 ± 0.032 d	0.133 ± 0.004 d	0.676 ± 0.676 d	0.103 ± 0.008 d
	300	0.626 ± 0.008 e	0.154 ± 0.001 e	0.779 ± 0.779 e	0.117 ± 0.001 e
12	0	0.518 ± 0.045 a	0.122 ± 0.012 a	0.640 ± 0.056 a	0.107 ± 0.009 a
	1	0.529 ± 0.061 a	0.118±0.012 a	0.648 ± 0.074 a	0.113 ± 0.012 a
	10	0.561 ± 0.042 a	0.129 ± 0.008 a	0.690 ± 0.050 a	0.120 ± 0.008 a
	100	0.595 ± 0.022 a	0.134 ± 0.003 a	0.729 ± 0.025 a	0.137 ± 0.004 b
	300	0.719 ± 0.016 b	0.178 ± 0.003 b	0.897 ± 0.013 b	0.150 ± 0.008 b

Values are means ± SE (P<0.05, n=5). Chl*a*, chlorophyll *a*; Chl*b*, chlorophyll *b*; Chl (*a* + *b*), sum of chlorophyll *a* and *b*; Car, carotenoid.
Los valores son promedios ± EE (P<0,05; n=5). Chl*a*, clorofilia *a*; Chl*b*, clorofilia *b*; Chl (*a* + *b*), suma de lãs clorofilas *a* y *b*; Car, carotenoides.

increased from 10:00 to 12:00 h. After 16:00 h, the net photosynthetic rate of all materials declined to the lowest level; no significant differences in this parameter were detected among the different treatments.

Stomatal conductance. Stoma is the channel of carbon dioxide exchange for photosynthesis, and the closing or opening of this channel affects leaf photosynthesis and transpiration. Different from those of the net photosynthetic rate, the peaks of stomatal conductance were observed at 10:00 h, with 1.09, 1.13 (14:00 h), and 1.61 H_2O mmol/m²/s of the control after 4, 8, and 12 d, respectively. In addition, the stomatal conductance of *A. cepa* var. *agrogarum* negatively correlated with Cd concentration. The minimum stomatal conductance of all treatments was observed at 14:00 h and appeared similar to the net photosynthetic rate.

Transpiration rate. The transpiration rate of *A. cepa* var. *agrogarum* functional leaves varied with Cd concentration and treatment duration. The transpiration rate decreased after 4 d to 12 d of treatment, and then remained consistent with the remaining treatment time. In addition, the stomatal conductance of the *Allium* plants negatively correlated with Cd concentration.

Whole-plant toxicity of Cd^{2+} and ICP test. Exposure of the *Allium* plants to different concentrations of Cd^{2+} for 12 d led to significant Cd^{2+} concentration in the roots and leaves of the *Allium* plants (Fig. 6a and 6b). As shown in Figures 6a and 6b, the Cd concentration significantly increased in roots and leaves exposed to Cd in comparison with values in the control. In particular, the Cd concentration in roots and leaves peaked, with 4435.8 ± 20.2 and 50.5 ± 0.3 μg/g DW, after 12 d of treatment with 300 μM Cd.

The concentration of Cd in leaves and roots increased with increasing Cd concentration in the solutions. The metal was largely restricted to roots, with a slight amount being transported to aerial parts. The Cd concentrations in the leaves were 39.9 ± 0.6, 5.7 ± 0.2, and 1.5 ± 0.03 μg/g DW after exposure to 100, 10, and 1 μM, respectively. Meanwhile, the leaf/root ratios were 0.76%, 0.74%, 1.2%, and 1.1% after exposure to 1, 10, 100, and 300 μM Cd, respectively (Fig. 6a and 6b).

The effects of Cd on essential minerals for *Allium* plants are displayed on Fig. 6 in panels c to l. The concentrations of Ca in roots and leaves (Fig. 6c and 6d) presented a similar trend to those of Zn (Fig. 6k and 6l) after treatment with the different concentrations of Cd: the Ca and Zn concentrations significantly increased on roots (P<0.05), except for the Ca concentration at 1 μM Cd, while they significantly decreased (P<0.05) in the leaves (Fig. 6c, 6d, 6k, and 6l) as Cd concentrations increased in the nutrient solution. Increasing concentrations of Cd treatments significantly decreased

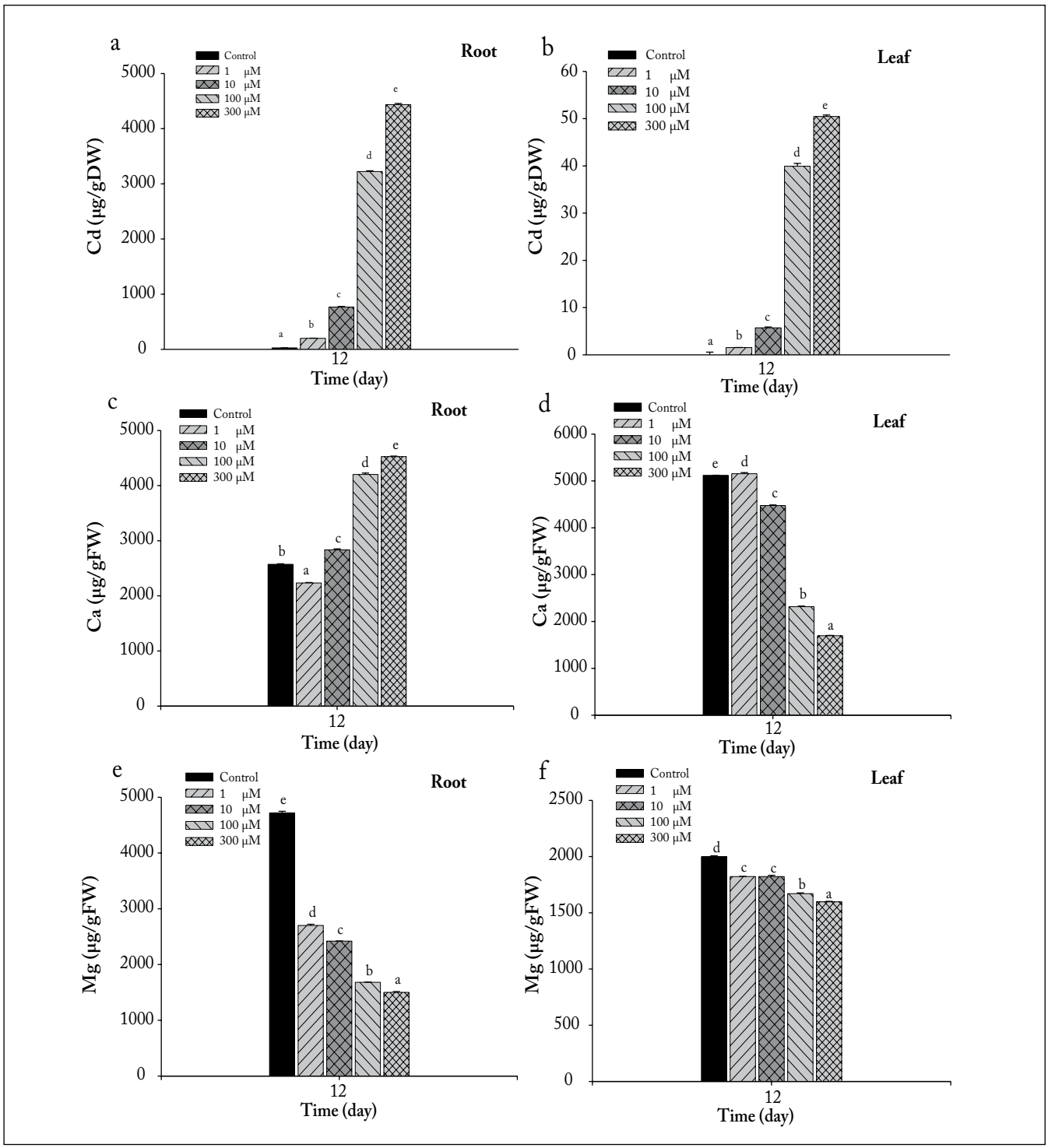

Fig. 6. Concentration of Cd and several minerals in roots and leaves of *Allium cepa* var. *agrogarum* L. after treatment with different concentrations of Cd^{2+} for 12 days. (a) Cd in roots, (b) Cd in leaves, (c) Ca in roots, (d) Ca in leaves, (e) Mg in roots, (f) Mg in leaves, (g) Fe in roots, (h) Fe in leaves, (i) Mn in roots, (j) Mn in leaves, (k) Zn in roots, (l) Zn in leaves. Vertical bars denote SE. Values with different letters differ significantly from each other (P<0.05, t-test).

Fig. 6. Concentración de Cd y varios minerales en raíces y hojas de *Allium cepa* var. *agrogarum* después del tratamiento con varias concentraciones de Cd^{2+} por 12 días. (a) Cd en raíces, (b) Cd en hojas, (c) Ca en raíces, (d) Ca en hojas, (e) Mg en raíces, (f) Mg en hojas, (g) Fe en raíces, (h) Fe en hojas, (i) Mn en raíces, (j) Mn en hojas, (k) Zn en raíces, (l) Zn en hojas. Las barras verticales son el EE. Los valores con letras diferentes son estadísticamente diferentes (P<0,05; t-test).

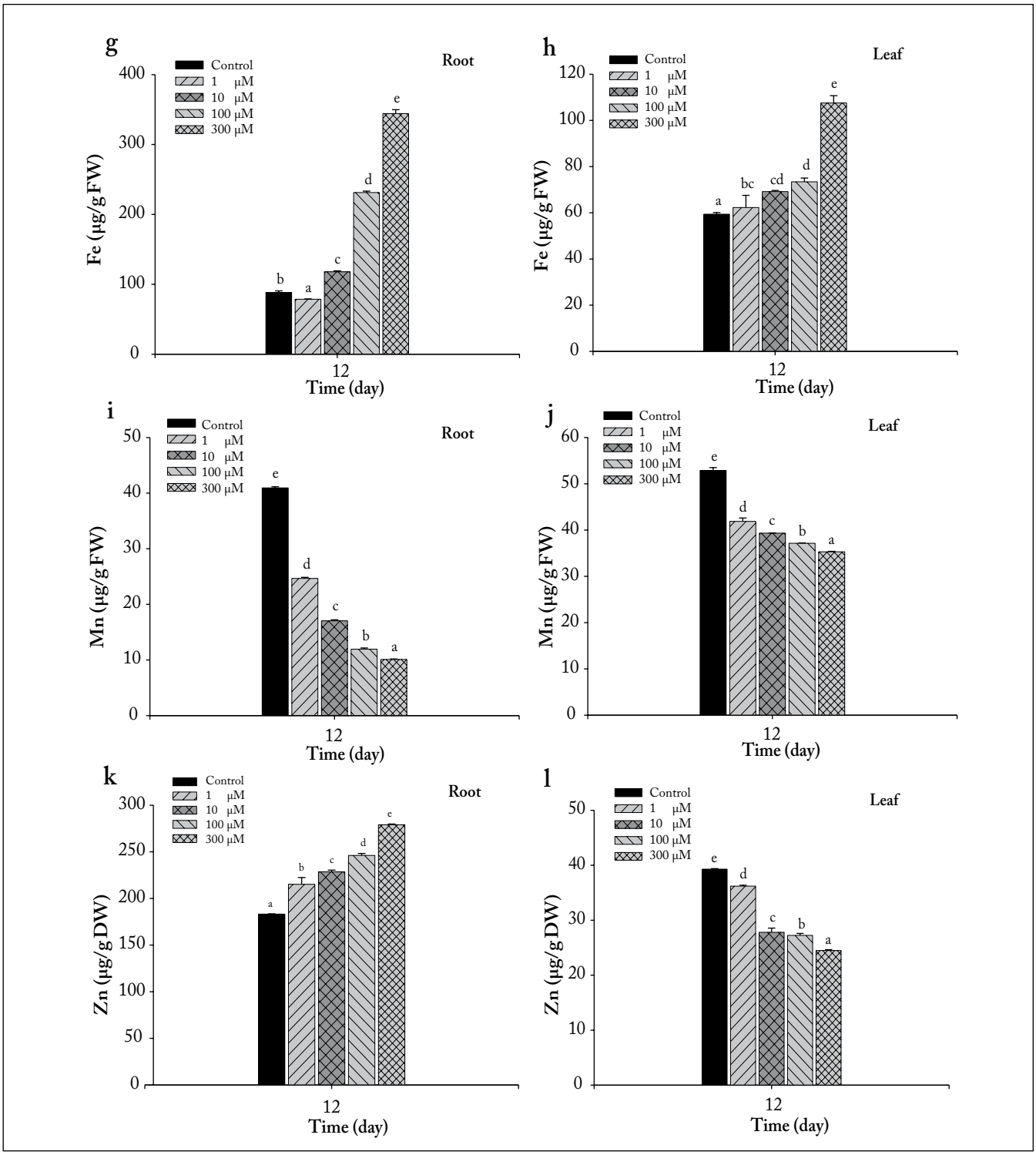

Fig. 6. Content of Cd and several minerals in roots and leaves of *Allium cepa* var. *agrogarum* L. after treatment with different concentrations of Cd2+ for 12 days. (a) Cd in roots, (b) Cd in leaves, (c) Ca in roots, (d) Ca in leaves, (e) Mg in roots, (f) Mg in leaves, (g) Fe in roots, (h) Fe in leaves, (i) Mn in roots, (j) Mn in leaves, (k) Zn in roots, (l) Zn in leaves. Vertical bars denote SE. Values with different letters differ significantly from each other (P<0.05, t-test).

Fig. 6. Content of Cd and several minerals in roots and leaves of *Allium cepa* var. *agrogarum* L. after treatment with different concentrations of Cd2+ for 12 days. (a) Cd in roots, (b) Cd in leaves, (c) Ca in roots, (d) Ca in leaves, (e) Mg in roots, (f) Mg in leaves, (g) Fe in roots, (h) Fe in leaves, (i) Mn in roots, (j) Mn in leaves, (k) Zn in roots, (l) Zn in leaves. Vertical bars denote SE. Values with different letters differ significantly from each other (P<0.05, t-test).

tissue Mg and Mn concentrations (P<0.05), except for Mg concentration at 10 μM Cd (Figs. 6e, 6f, 6i, and 6j). The Fe level significantly increased in roots and leaves with increasing Cd concentrations (P<0.05; Fig. 6g and 6h).

DISCUSSION

Strong net Cd^{2+} influx in roots indicates considerable potential for Cd enrichment. In this study, the spatial and temporal kinetics of the net Cd^{2+} flux were examined in the roots of *A. cepa* var. *agrogarum* L. by using an NMT that is highly sensitive to Cd^{2+} movement (Fig. 1). Previous studies applied a similar method to detect Cd^{2+} flux along the roots of monocotyledonous plants (Farrell et al., 2005; Pineros et al., 1998). The Cd^{2+} influx in the roots of wheat (*Triticum aestivum* cv Grandin) exposed to 50 μM Cd^{2+} peaks (0.28 pmol $Cd^{2+}/cm^2/s$ to 0.35 pmol $Cd^{2+}/cm^2/s$) in the region of 0.6 mm to 1.2 mm from the root tips (Pineros et al., 1998). Similar positional effects were observed along the roots of other herbaceous plants exposed to Cd stress (Pineros et al., 1998; Farrell et al., 2005). In the roots of the dicotyledonous plant *P. canescens*, the Cd^{2+} influx peaked in the apex region (0 mm to 0.9 mm from root tip) (He et al., 2011). However, the use of this technique in a common dicotyledonous plant has yet to be reported.

These data indicate that the spatial patterns of the net Cd^{2+} flux along the roots are similar in dicotyledonous plants but probably different between monocots and dicotyledonous plants. The reason for this trend in net Cd^{2+} flux remains unclear. Further experiments should focus on examining whether or not the root anatomy or localization of the Cd^{2+} uptake system varies between these plant groups. Continuous increases in Cd concentration in the leaves and roots after exposure for 12 d (Fig. 6) suggest that a long period elapses for *A. cepa* var. *agrogarum* L to reach the saturation of the net Cd^{2+} influx. Moreover, the *Allium* plants still grew under the current experimental conditions. Overall, these data imply that *A. cepa* var. *agrogarum* L. presents a great potential for Cd enrichment.

Cd accumulation impairs tissue-specific oxidative stress and photosynthesis of *A. cepa* var. *agrogarum* L. Cd is considered a class 1 human carcinogen by the International Agency for Research on Cancer (IARC, 1993). Cd pollution presents a significant environmental problem that affects numerous physiological and biochemical processes; its effects include alterations in photosynthetic rates, photosynthetic pigments, chlorophyll fluorescence, and nutrient homeostasis in plants (López-Millán, 2009). Plants have developed complex mechanisms to minimize the damage from exposure to nonessential metals (Gallego et al., 2012). In the present investigation, the root lengths of *A. cepa* var. *agrogarum* L. decreased with increasing Cd concentration and prolonging exposure time. In addition, treatment with 1 μM Cd promoted root growth. This result agrees with previous findings (Liu et al., 2008).

Under most environmental conditions, Cd^{2+} initially enters the roots and then it is transported to shoots via xylem (Uraguchi et al., 2009). As a result, the root is the first, most sensitive and accessible part to Cd^{2+} toxicity. One of the major functions of roots includes nutrient uptake. Heavy metal micronutrients, such as Ca, Mg, Fe, Mn, Mo, Ni, and Zn, perform essential functions in plant cell growth and development (Blomster et al., 2011). To illustrate, Zn is a cofactor of numerous enzymes, through which the metal is involved in protein binding, enzyme activity mediation, transcriptional and translational regulation, and signal transduction (Appenroth, 2010). Mg and Mn perform similar physiological functions in plants. However, the deficiency in essential micronutrients due to the increased contents of photosynthetic pigments inhibited the photosynthetic efficiency, stomatal conductance, and transpiration of *A. cepa* var. *agrogarum* L. (P<0.05; Fig. 6 and Table 1).

In consideration of the importance of essential micronutrients in plant physiology, maintaining homeostasis of these heavy metals in plant cells is essential. We propose that Cd^{2+} toxicity is involved in impairing the uptake and transport of these nutrient elements, thereby disturbing ion homeostasis in vascular plants. Cd chemically resembles Zn and Fe. To date, a Cd-specific influx transporter has yet to be found in plant cells. In addition, the uptake of Cd is likely to occur through available metal uptake ZIP transporters (or alike) that present high specific transport affinity for Zn or Fe but low affinity for Cd (Korshunova et al., 1999; Pence et al., 2000). Thus, the uptake of Cd in root cells appears to be an opportunistic event. The family of NRAMP (natural resistance-associated macrophage proteins) metal ion transporters represents another important group of transmembrane proteins involved in metal transport and homeostasis. These transporters are expressed in both roots and shoots, and are considered to be "general metal ion transporters" because of their capability to transport Mn^{2+}, Zn^{2+}, Cu^{2+}, Fe^{2+}, Cd^{2+}, Ni^{2+}, and Co^{2+} (Nevo & Nelson, 2006). These transporters may explain the mechanism by which Cd^{2+} is absorbed and concentrated in roots of *A. cepa* var. *agrogarum* L., and the reason why the concentration of Zn and Fe in the roots is proportional to the increase in Cd concentration. In particular, Cd^{2+} uptake occurs through transmembrane carriers engaged in the uptake of Ca^{2+}, Fe^{2+}, Mg^{2+}, Cu^{2+}, and Zn^{2+} (Clemens, 2006; Roth et al., 2006). Several of these metals can inhibit Cd uptake from the rhizospheric solution and Cd accumulation in plant roots (Cataldo et al., 1983; Costa & Morel, 1993; Hart et al., 2002; Zhao et al., 2002, 2006). Ca levels affect Cd uptake because Cd competes with Ca for Ca channels (Perfus-Barbeoch et al., 2002; Wojas et al., 2007). Li et al. (2012) indicated that Cd may permeate the channels and bind transiently to a site in the pore, reversibly obstructing the passage of Ca^{2+}. Zn and Cd most possibly cross the plasma

membrane via members of the ZIP transporter family (ZRT-IRT-like protein; Zinc-regulated transporter, Iron-regulated transporter protein). Zn excess often evokes an Fe-deficiency response, with the induced expression of Fe uptake transporters such as IRT1. This route could explain the similar changes in the contents of Ca, Zn, and Fe to Cd.

The accumulation of O_2^- and H_2O_2 in the roots and leaves of Cd-treated *Allium* plants indicates that Cd exposure leads to oxidative stress, as reported in several studies (Schutzendubel et al., 2001, 2002; Schutzendubel & Polle, 2002; Romero-Puertas et al., 2004; Garnier et al., 2006; Rodriguez-Serrano et al., 2006, 2009). Our data suggest that *Allium* roots, which showed considerable Cd accumulation, presented strong O_2^- and H_2O_2 accumulation. In contrast to roots, leaves accumulated comparatively lower Cd (Fig. 6). However, the two organs presented similar changes in the levels of O_2^- and H_2O_2 (Fig. 4). As is well-known, ROS perform a dual function in metal stress response. That is, ROS act both as oxidative molecules, aggressively reacting with cellular macromolecules, and as signal transduction molecules (Sandalio et al., 2012). For example, H_2O_2 overproduction leads to serious oxidative damage, and thus threatens cellular function. However, H_2O_2 is also an important signaling molecule that regulates plant development, hormone signaling, programmed cell death, and stress response and tolerance (Matilla-Vazquez & Matilla, 2012). Thus, controlling ROS generation in plant cells during metal exposure is important to maintain developmental processes and general stress responses. ROS levels substantially rise in heavy metal-sensitive plant species if sufficient antioxidant enzymes are unavailable. In consequence, ROS-induced cellular damage induces local programmed cell death, and generally affects plant growth and development. For example, Cd-induced ROS generation can activate auxin oxidase, which degrades auxin, and change the auxin-regulated morphogenetic response in *Arabidopsis thaliana* rosette leaves (Blomster et al., 2011; Elobeid & Polle, 2012). The effect of this phenomenon on photosynthesis includes restraining photosystem II (PSII) activity, inhibiting PSII photoreaction, lowering photophosphorylation, reducing the activity of chloroplast enzymes RuBPC and phosphoribulokinase, decreasing photosynthetic pigments (e.g., total chlorophyll content and Chl *a/b* ratio), diminishing net photosynthesis in leaves, and reducing chloroplast metabolism (Clijsters et al., 1985).

Our results showed that the photosynthetic efficiency, stomatal conductance, and transpiration of *A. cepa* var. *agrogarum* L. correlated negatively with Cd concentration and were significantly inhibited ($P < 0.05$). In addition, the results of the present study are consistent with previous reports (Greger & Ögren, 1991; Krupa et al., 1993; Ciscato et al., 1999; Larbi et al., 2002). Finally, Cd induced the production of H_2O_2, which acts as a signaling molecule to trigger the expression of the WRKY75, Zat11, and NAM transcription factors that stimulate programmed cell death in plants (Gechev & Hille, 2005). Large amounts of toxic metals enter plant cells, which possess no sufficient mechanisms to detoxify these metals. Thus, excess metals induce ROS accumulation, which further negatively influences plant growth and development, suppresses photosynthesis, damages nucleic acids and proteins, enhances programmed cell death, and induces senescence.

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